University of Alberta

Molecular Detection and Identification of Phytoplasmas and Establishment of Phytoplasma-free Clonal Plants

by

Keri Wang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Plant Science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 1997

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-22688-3

Canada

University of Alberta

Library Release Form

Name of Author: Keri Wang

Title of Thesis: Molecular Detection and Identification of Phytoplasmas and Establishment of Phytoplasma-free Clonal Plants

Degree: Master of Science

Year this Degree Granted: 1997

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Keri Wang

Lixin Village, Langan Town

Huaibin County, Henan Province

P. R. China 464411

Aug 29. 1997

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of graduate Studies and Research for acceptance, a thesis entitled **Molecular Detection and Identification of Phytoplasmas and Establishment of Phytoplasma-free Clonal Plants** submitted by **Keri Wang** in partial fulfillment of the requirements for the degree of **Master of Science** in **Plant Science.**

Jalpa P. Tewari (Supervisor)

Francis Yeh

Walter Dixon

Chuji Hiruki

July 23, 1997

To

My Parents

ABSTRACT

Polymerase chain reaction (PCR) was found to be the most sensitive method for detection of phytoplasmas requiring as little as 16 pg of total nucleic acid extracted from woody host plants. The concentration of phytoplasmas in tissue cultures was significantly higher than that in plants grown in the greenhouse. Short-duration microwave heating did not affect the structure of DNA and was very useful for preserving phytoplasma DNA in tissues for molecular analyses and international exchange of research specimens. Paulownia witches'-broom (PaWB) phytoplasma isolates were very closely related to each other when collected from diverse geographic sites, but were different from jujube witches'-broom (JWB) phytoplasma isolates on the basis of restriction fragment length polymorphism and 16S rDNA sequence analyses. PaWB isolates from China, Japan, and South Korea share the same 16S rRNA gene sequences and JWB phytoplasmas from these countries also share the same 16S rRNA gene sequences. Monarda yellows was, for the first time, identified to be associated with a phytoplasma which belongs to the aster yellows group through molecular analyses. Phytoplasma-free plantlets were established by PCR-based screening of meristem cultures of PaWB phytoplasma-infected paulownia after subjecting them to heat treatment at 35°C for 5 weeks.

ACKNOWLEDGEMENTS

There are many people who helped me during the course of my research. Most of all, I would like to thank my research supervisor, Dr. C. Hiruki. Under his personal guidance, I initiated and concluded this research. His advice, assistance and support have been instrumental in the preparation and completion of this thesis. I wish to thank my supervisor, Dr. J. P. Tewari, for his encouragement and valuable guidance throughout the course of this project. They have created opportunities and possibilities for me that I would have never realized without them.

Dr. Francis Yeh and Dr. Walter Dixon, members of my supervisory committee, have helped me in many ways, providing me with useful advice and valuable opportunities for discussing my research project.

Mrs. Shirley Brezden has always been helpful in the laboratory with warm friendship and useful technical assistance at various stages of this study.

My parents, Fengde Wang and Jianlan Chu, were a source of strength for me. They taught me to enjoy hard work, to achieve a balance in everything I do, and to never forget the spiritual aspects in my daily life.

Most importantly, I am very grateful to my wife, Aixia Wang, for her continuous support, encouragement and patience throughout my graduate program. She has sacrificed a lot in order to let me pursue my academic studies and research. None of my achievements would have been possible without her.

TABLE OF CONTENTS

CHAPTER		PAGE
I	LITERATURE REVIEW	1
I.1	INTRODUCTION	2
I.2	GENERAL PROPERTIES OF PHYTOPLASMAS	3
I.3	MAINTENANCE OF PHYTOPLASMAS	5
I.4	DETECTION AND IDENTIFICATION OF PHYTOPLASMAS	8
I.4.1	Electron Microscopy	8
I.4.2	Histochemistry	9
I.4.3	Immunology	10
I.4.3.1	*Monoclonal Antibodies*	11
I.4.3.2	*Enzyme-linked Immunosorbent Assay (ELISA)*	12
I.4.3.3	*Dot Blot Immunoassay*	13
I.4.3.4	*Immunofluorescence Microscopy*	14
I.4.4	Molecular Biology	14
I.4.4.1	*Nucleic Acid Hybridization*	15
I.4.4.2	*Polymerase Chain Reaction (PCR)*	17
I.4.4.3	*Restriction Length Fragment Polymorphism (RFLP)*	19
I.4.4.4	*Heteroduplex Mobility Assay (HMA)*	20
I.5	ETIOLOGY OF PHYTOPLASMA DISEASES	21
I.6	TAXONOMY OF PHYTOPLASMAS	24

I.7 CONTROL OF PHYTOPLASMA DISEASES 27

I.8 OBJECTIVES OF THIS STUDY 28

I.9 REFERENCES 32

II. DNA AMPLIFICATION BASED ON POLYMERASE CHAIN REACTION FOR SENSITIVE DETECTION OF PHYTOPLASMA ASSOCIATED WITH PAULOWNIA WITCHES'-BROOM 51

II.1 INTRODUCTION 52

II.2 MATERIALS AND METHODS 53

II.2.1 Sources of Healthy and Diseased Paulownia Plants 53

II.2.2 Extraction of Nucleic Acids from Plants 54

II.2.3 PCR Primers 55

II.2.4 PCR Amplification 55

II.2.5 Agarose Gel Electrophoresis of PCR Products 56

II.3 RESULTS AND DISSCUSSION 56

II.4 REFERENCES 63

III. PRESERVATION OF PHYTOPLASMA DNA IN MICROWAVE-DRIED TISSUES 65

III.1 INTRODUCTION 66

III.2 MATERIALS AND METHODS 67

III.2.1 Phytoplasma-infected Plant Tissues 67

III.2.2 Microwave-treatment and Air-drying of Sample Tissues 68

III.2.3 DNA Extraction 68

III.2.4 DNA Amplification 69

III.2.5 Restriction Fragment Length Polymorphism (RFLP) 69

III.2.6 DNA Sequencing 70

III.3 RESULTS AND DISCUSSION 70

III.4 REFERENCES 82

IV. GENETIC CHARACTERIZATION OF PHYTOPLASMA ISOLATES FROM PAULOWNIA AND JUJUBE IN IN DIFFERENT GEOGRAPHIC AREAS 84

IV.1 INTRODUCTION 85

IV.2 MATERIALS AND METHODS 87

IV.2.1 Sources of Healthy and Diseased Plants 87

IV.2.2 Extraction of Nucleic Acids from Plants 87

IV.2.3 Primers and PCR Conditions 87

IV.2.4 Nested-PCR 88

IV.2.5 RFLP Analyses of Nested-PCR Products 88

IV.2.6 Sequencing of the Amplified Fragments 89

IV.3 RESULTS 90

IV.3.1 Amplification of 16S rDNA Sequences of Phytoplasmas 90

IV.3.2 RFLP Analysis 90

IV.3.3 Identification of JWB Phytoplasma 90

IV.3.4 Sequencing Analysis of the Amplified Fragment 91

IV.4 DISCUSSION 92

IV.5 REFERENCES 103

V. MONARDA YELLOWS, A NEW DISEASE ASSOCIATED WITH A PHYTOPLASMA OF THE ASTER YELLOWS SUBCLADE 107

V.1 INTRODUCTION 108

V.2 MATERIALS AND METHODS 109

V.2.1 Plants and Phytoplasma Strains 109

V.2.2 DNA Extraction 109

V.2.3 Primer Pairs and PCR Conditions 109

V.2.4 Nested-PCR Assays with Two Universal Primer Pairs 110

V.2.5 RFLP Analyses of PCR Products 110

V.2.6 Electron Microscopy 111

V.3 RESULTS 111

V.3.1 Disease Symptoms 111

V.3.2 Amplification of 16S rDNA Sequences and Nested-PCR 112

V.3.3 RFLP Analysis of 16S rDNA of Phytoplasmas 112

V.3.4 Electron Microscopy 113

V.4 DISCUSSION 113

V.5 REFERENCES 120

VI. PCR (POLYMERASE CHAIN REACTION)-BASED SELECTION OF PHYTOPLASMA-FREE CLONES OF PAULOWNIA TISSUE CULTURE AFTER HEAT TREATMENT OF WITCHES'-BROOM 122

VI.1 INTRODUCTION 123

VI.2 MATERIALS AND METHODS 124

VI.2.1 Sources of Healthy and Phytoplasma-infected Paulownia Tissue Cultures 124

VI.2.3 Heat Treatment and Meristem Culture 125

VI.2.4 DNA Isolation 125

VI.2.5 PCR Detection 126

VI.3 RESULTS AND DISCUSSION 126

VI.4 REFERENCES 134

VII GENERAL DISCUSSION AND CONCLUSIONS 136

VII.1 GENERAL DISCUSSION 137

VII.2 CONCLUSIONS 142

VII.3 FUTURE PERSPECTIVES 144

VII.4 REFERENCES 146

LIST OF TABLES

Table I.1 Phytoplasma-disease groups based on relatedness of pathogens 30

Table VI.1 The number of phytoplasma-free clones of paulownia tissue cultures obtained through PCR-based screening after heat treatment of witches'-broom 133

LIST OF FIGURES

Fig. I.1 Phylogenetic tree constructed by parsimony analysis of 16S rDNA sequences from 30 phytoplasmas, two *Acholeplasma* spp., and two *Anaeroplasma* spp., employing A. *Abactoclasicum* as the outgroup. 31

Fig. II.1 Polymerase chain reaction analysis of DNA extracted from healthy paulownia plants, tissue culture of witches'-broom affected paulownia and paulownia plants showing witches'-broom symptoms. 59

Fig. II.2 Comparison of phytoplasma occurrence in phytoplasma-infected tissue culture maintained at 25-28 °C and in phytoplasma-infected paulownia plants grown at 23-25 °C under greenhouse conditions. 61

Fig. III.1 Agarose gel (1% w/v) electrophoresis of total DNA samples extracted from infected fresh paulownia tissue cultures, microwave-treated tissue cultures and midribs, air-dried tissue cultures and midribs. 73

Fig. III.2 PCR amplification of 16S rDNA fragment (1.2 kb) from genomic DNAs extracted from microwave-treated, fresh healthy and phytoplasma-infected paulownia tissues using a primer pair R16R2/F2. 75

Fig. III.3 Restriction fragment length polymorphism analysis. A 5 µl aliquot of PCR products was digested with *Alu*I (upper) and *Hha*I (lower) enzymes and separated in 5% polyacrylamide gel electrophoresis. 77

Fig. III.4 Sequencing results produced from amplified DNA from microwave-treated tissue cultures (20 min) and from untreated paulownia tissues showing typical witches'-broom symptoms. 79

Fig. III.5 Relationship between dehydration time and the dry weight of paulownia tissue culture after microwave heat-treatment. ····· 81

Fig. IV.1 Nested-PCR amplified 1.2 kb 16S rDNA fragments from DNA preparations from PaWB phytoplasma-infected paulownia leaves and from JWB phytoplasma-infected jujube leaves collected from China, Japan, and Korea. ····· 95

Fig. IV.2 Restriction fragment length polymorphism analysis of nested-PCR amplified 1.2 kb 16S rDNA fragments from PaWB, JWB, AY27, EY, PWB, CP, and AWB phytoplasma isolates using a primer pair R2/F2. ····· 97

Fig. IV.3 Autoradiograph of a polyacrylamide/urea gel showing sequencing reaction products produced by using 1.5 kb DNA fragment amplified from total DNA isolated from paulownia witches'-broom-affected paulownia and jujube witches'-broom-affected jujube plants collected in the field. ····· 99

Fig. IV.4 Nucleotide sequences of 16S rRNA genes of paulownia witches'-broom and jujube witches'-broom phytoplasmas and the corresponding DNA region of *Oenothera* phytoplasma. ····· 101

Fig. V.1 The symptoms of a monarda yellows-affected monarda shoot showing small leaves, leaf yellowing, and proliferation (A) compared with a healthy plant (B). ····· 115

Fig. V.2 16S rDNA fragment (1.2 kb) of phytoplasmas amplified by nested-PCR from various phytoplasma isolates. ····· 116

Fig. V.3 RFLP profiles of 16S rDNA amplified by nested-PCR from representative phytoplasma strains. ····· 118

Fig. VI.1 Phytoplasma-infected paulownia tissue culture maintained in a growth chamber for 4 weeks at 25 °C showing typical phytoplasma symptoms such as little leaves, chlorosis and abnormally activated axillary buds. 129

Fig. VI.2 Paulownia meristem culture maintained at 25°C after heat-treatment for 5 weeks at 35 °C. 130

Fig.VI.3 Polymerase chain reaction (PCR) detection of phytoplasma in heat-treated and untreated PaWB-affected paulownia tissue cultures. 131

LIST OF ABBREVIATIONS

AWB	Alfalfa witches'-broom
AY	Aster yellows
AYHB	Belgium hydrangea aster yellows
AYHF	French hydrangea aster yellows
bp	Basepair
CP	Clover proliferation
CTAB	cetyltrimethyl-ammonium bromide
dNTP	Deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)
EAY	Eastern aster yellows
EDTA	Ethylene diaminetetraacetic acid, disodium salt
EY	Elm yellows
hr	Hour(s)
JWB	Jujube witches'-broom
kb	Kilobase
MLO	Mycoplasmalike organism
SDS	Sodium dodecyl sulfate
MS	Murashige & Skoog
MY	Monarda yellows
NAA	α-naphthaleneacetic acid
NTP	Nucleotide triphosphate

PaWB	Paulownia witches'-broom
PCR	Polymerase chain reaction
PVP	polyvinylpyrrolidone
PWB	Potato witches'-broom
RFLP	Restriction fragment length polymorphism
rDNA	Ribosomal RNA gene
rRNA	Ribosomal RNA
TE	10mM Tris·HCl, 0.1mM EDTA, pH 8.0
U	Unit
UV	Ultraviolet
W/V	Weight/volume

CHAPTER I

LITERATURE REVIEW

I.1 INTRODUCTION

The first mention of an association between wall-less prokaryotes and plants was reported in 1967. Japanese scientists, Dr. Doi and his colleagues (1967), discovered cell wall-less prokaryotes resembling mycoplasmas in the phloem of plants with mulberry dwarf, potato witches'-broom, aster yellows and paulownia witches'-broom by electron microscopy. The disease symptoms of infected plants could be eliminated and such microorganisms were observed to disappear temporarily as long as treatments with tetracyclines lasted, but penicillin treatments had no effect. Because mycoplasmas were known to be sensitive to tetracyclines (Ishiie *et al.*, 1967; Davis *et al.*, 1968) but not to penicillin (Whitecomb and Black, 1982), whereas the PLT (psittacosis-lymphogranuloma-trachoma) agents were affected by both of these antibiotics, the microorganisms were considered mycoplasma-like but not chlamydia-like and thus were called mycoplasma-like organisms (MLOs) on the basis of their morphology and ultrastructure (Doi *et al.*, 1967). Subsequently, mycoplasma-like organisms were found to be associated with many yellows diseases previously thought to be caused by viruses.

With the advent of molecular biology, accumulated evidence has indicated that plant pathogenic MLOs are significantly different from animal mycoplasmas in several aspects and were renamed "phytoplasmas" at the 10th International Congress of the International Organization for Mycoplasmology (IOM) in 1994. At least four types of molecular data (DNA base composition, genome size, ribosomal protein genes and membrane properties) support the placement of phytoplasmas in the class *Mollicutes*. Although the inability so far to culture phytoplasmas in artificial medium has hindered

research on their classification and characterization, significant progress in various areas of phytoplasmas study, as described below, has been made through the application of molecular biological techniques.

I.2 GENERAL PROPERTIES OF PHYTOPLASMAS

Phytoplasmas are cell wall-less prokaryotes. They are bounded by a "unit" membrane, and have cytoplasm, ribosomes, and both DNA and RNA. In ultrathin sections, they appear as a complex of multibranched, beaded, filamentous or spheroidal polymorphic bodies ranging from 175-400 nm in diameter for the spherical and oblong cells and up to 1700 nm long for the filamentous forms (Florence and Cameron, 1978; Waters and Hunt, 1980). Phytoplasmas are generally present in a small number of phloem sieve tubes and in the salivary glands of insect vectors (McCoy, 1983). While phytoplasmas are assumed to multiply in the phloem, little is known about its mechanism. Most phytoplasmas are transmitted from plant to plant by leafhoppers in a persistent manner (Sinha and Chiykowski, 1967; Purcell, 1982), but some are transmitted by psyllids and planthoppers. Phytoplasmas also grow in the alimentary canal, hemolymph, salivary glands, and intracellularly in various body organs of their insect vectors (Purcell, 1982). Numerous attempts to culture phytoplasmas on artificial nutrient media or cell-free media have been unsuccessful so far (Lee and Davis, 1986). At present, phytoplasmas are believed to cause more than three hundred different diseases among diverse plant species including fruit trees, forest and ornamental trees, grasses, vegetables, flowers and agricultural crops (McCoy *et al.*, 1989). The severity and nature of disease symptoms

depend on the plant and the type of phytoplasma agent. Some plants may show no symptoms for years, even though phytoplasmas are abundant in their tissues. In other cases, plants deteriorate rapidly and die even though the phytoplasmas never become very abundant. Disease symptoms vary with plants, and include yellowing, dwarfing, virescence, phyllody, proliferation, witches'-broom, shortening of internodes, stunting, decline etc. Phytoplasma titers in infected plants are generally low (Kollar *et al.*, 1990) and in a state of dynamic change as shown by data of serological testing (Sinha and Benhamou, 1983) and nucleic acid hybridization (Kuske and Kirkpatrick, 1989; Deng and Hiruki, 1990b). Recently, significant progress in detection, identification and phylogenetic studies of phytoplasmas has been made since the introduction of molecular biological techniques. The guanine-plus cytosine (G+C) content of several phytoplasmas has been estimated to range from 23 to 29% (Razin, 1992) by analyzing the hydrolyzed phytoplasma DNAs using high-performance liquid chromatography (Kollar and Seemüller, 1989). Phytoplasma genome analysis has also been performed on a number of isolates using pulsed field gel electrophoresis, and field inversion gel electrophoresis (Davis *et al.*, 1990; Neimark and Kirkpatrick, 1991). Sequence data of 16S rRNA genes of several phytoplasmas indicated that phytoplasmas are evolutionarily most closely related to *Acholeplasma* and *Anaeroplasma* (Kirkpatrick and Fraser, 1989; Lim and Sears, 1989; Sears and Kirkpatrick, 1994). The estimated sizes of phytoplasma genomes vary from 450 to 1180 kb (Davis *et al.*, 1990; Neimark and Kirkpatrick, 1991; Lim and Sears, 1991; Sears and Kirkpatrick, 1994). A variety of molecular techniques including full-length 16S rDNA sequence analysis, specific 16S-23S rDNA spacer sequences, and polymerase chain reaction (PCR) amplification with specific primers have been used to

examine many phytoplasma strains (Deng and Hiruki, 1990a, b, c, 1991a, b; Lee *et al.*, 1993b; Namba *et al.*, 1993b; Gundersen *et al.*, 1994a, b; Seemüller *et al.*, 1994). Eleven distinct phytoplasma 16S rRNA genome groups and more than 25 subgroups have been identified on the basis of the results of restriction fragment length polymorphism (RFLP) analyses of PCR amplified 16S ribosomal DNA (rDNA) (Gundersen *et al.*, 1994b, 1996; Lee *et al.*, 1993b). Management of phytoplasmal diseases will be facilitated by insufficient understanding of phytoplasmas in the future. Although the pathogens are susceptible to tetracycline, the tetracycline treatment only suppresses the symptom development temporarily (Raju and Nyland, 1988). More detailed studies of the genome of phytoplasmas are expected to reveal the interrelationship between the gene structure and function, and lead to disease control.

I.3 MAINTENANCE OF PHYTOPLASMAS

Since phytoplasmas cannot be cultured *in vitro* (Chang and Chen, 1982; Lee and Davis, 1986), their maintenance has mainly relied on their continuous propagation in their specific host plants which has been difficult to achieve because maintenance by graft inoculation from diseased to healthy plants has forced problems of both irregular efficiency of inoculation and variations in phytoplasma titers due to environmental or seasonal fluctuations. In addition, as phytoplasmas are naturally transmitted by insect vectors, plants infected by a particular phytoplasma isolate have to be kept under insect-proof conditions to exclude cross-contamination. The species of insect vector, the source plant of phytoplasmas, and the suitable growth stage of the plant need to be taken into

account to achieve high rates of insect transmission of phytoplasmas (Jensen, 1956; Chiykowski and Sinha, 1982). Moreover, maintaining a continuous supply of insects and plants for long periods of time for maintaining phytoplasma isolates is troublesome (Chiykowski, 1977, 1988). Furthermore, Southern blot analysis (Ahrens *et al.*, 1993) and 16S rDNA analysis (Denes and Sinha, 1992; Schneider *et al.*, 1993) revealed that most of periwinkle-maintained isolates with a few exceptions are genetically different from those found on naturally infected plants.

To avoid the above mentioned problems, micropropagation of phytoplasma-diseased plant tissue cultures has been used for maintaining phytoplasmas in their original host plants. Potato witches'-broom (Petru and Ulrychova, 1975), aster yellows (Mitsuhashi and Maramorosch, 1964; Jacoli, 1974, 1978), poplar witches'-broom (Cousin *et al.*, 1990), evening primrose (Sears and Klomparens, 1989) and lethal yellowing phytoplasmas (Maia and Beck, 1976; McCoy 1978) have been maintained in shoot cultures. Phytoplasmas have been maintained in stable conditions and at high titers for several years in the aseptic leaf tip cultures of evening primrose (Sears and Kolmparens, 1989). The concentration of paulownia witches'-broom phytoplasma in tissue culture was significantly higher than that in plants grown under greenhouse conditions (Wang *et al.*, 1994). Micropropagation of *Malus pumila* has been used for maintaining apple proliferation (AP) phytoplasma for more than ten years (Jarausch *et al.*, 1996). Restriction fragment length polymorphism (RFLP) analysis of the amplified chromosomal DNA fragments revealed no genetic difference between the AP phytoplasma isolates. Eight years after culture initiation, diseased shoots still exhibited typical symptoms like witches'-broom, small leaves with large stipules and stunted growth (Jarausch *et al.*,

1996). However, in the case of tissue culture of clover with clover phyllody, the deletion of extrachromosomal DNA was reported (Denes and Sinha, 1992).

A cryopreservation method was developed to maintain isolates of aster yellows phytoplasmas for relatively long periods of time by using frozen whole leafhoppers (Chiykowski, 1977, 1983). A phytoplasma isolate preserved in whole *Macrosteles fascifrons* at -70 °C still had a high level of infectivity after six years. This method provides a relatively simple, economical and effective way to store infected plants or insect materials for future serological or genomic identification of the phytoplasmas.

Generally speaking, most of the above-mentioned methods are appropriate and useful, but they are time-consuming, expensive, and sometimes not suitable for international exchange of phytoplasma-infected materials. A simple, economic and practical method is required for preservation of phytoplasma DNA for future study. Microwave treatment has been reported to be useful for isolating DNA from fungi, plants, protists, animals, or paraffin-embedded tissue, and the isolated DNA has been used for successful amplification of ribosomal genes from these organisms (Goodwin and Lee, 1993; Hultuer and Cleaver, 1994; Banerjee et al., 1995; Wang *et al.*, 1995). The procedure is a time-saving (15 min) and cost-effective and helps to eliminate several sources of contamination that often associated with other DNA isolation methods. Although microwave treatment of clover proliferation (CP) phytoplasma in infected periwinkle plant tissues was reported to preserve CP phytoplasma DNA in microwave-dried tissues (Khadhair *et al.*, 1995), no molecular evidence was presented regarding the effect of a brief microwave treatment on phytoplasma DNA sequences.

I.4 DETECTION AND IDENTIFICATION OF PHYTOPLASMAS

Sensitive and accurate detection and identification of pathogens are a prerequisite for pathogen characterization and successful control of plant diseases. Diagnosis of phytoplasma-associated diseases is one of the most difficult aspects of research on plant diseases due to the inability to culture the pathogens *in vitro*. Initial diagnosis of phytoplasma-associated plant diseases and classification of phytoplasmas relied primarily on electron microscopy, DNA staining of sieve elements of diseased plants, and biological characteristics including phytoplasma-vector relationships, host ranges, and symptomology (McCoy *et al.*, 1989). However, this system is time-consuming, laborious and complicated, and often has resulted in misleading conclusions. Recent introduction of molecular technologies, however, has made significant progress in the rapid and accurate detection and identification as well as classification of phytoplasmas.

I.4.1 Electron Microscopy

Verification of phytoplasma involvement with any plant disease is ultimately dependent on the observation of phytoplasma bodies in plant tissues by transmission electron microscopy. Phytoplasmas in ultrathin sections appear as rounded to filamentous prokaryotic cells, 175 to 400 nm in diameter, that are bound by a single membrane (Chen and Hiruki, 1977; Waters and Hunt, 1980; McCoy, 1983). In most infected herbaceous plants, phytoplasmas may be observed in all organs such as roots, shoots, flowers, fruits, and leaves. However, phytoplasmas are usually more difficult to find in woody plants (McCoy *et al.*, 1989). Since some walled bacterial pathogens including rickettsia-like

organisms and bacteria-like organisms share the phloem habitat of plant (Saglio *et al.*, 1972), careful examination of electron-micrographs is required and the use of semithick sections is strongly recommended for examination (Chen *et al.*, 1989). Although electron-microscopy provides a direct method for detection of phytoplasmas, the characteristic pleomorphic morphology is of little help in the specific identification of these organisms, since there are numerous similar membranous bodies in crude plant extracts (Wolanski and Maramorosch, 1970).

I.4.2 Histochemistry

Some fluorochromes, such as acridine orange, ethidium bromide, and Hoechst 33258 nonspecifically bind to DNA forming a complex which exhibits a fluorescence and are used routinely to detect occurrence of phytoplasma in cell cultures (Chen, 1977; DelGiudice and Hopps, 1978; Steiner *et al.*, 1982; McGarrity *et al.*, 1983). Hoechst 33258 staining has been used to examine leafhopper cell lines infected with phytoplasmas and spiroplasmas (Steiner *et al.*, 1982, 1983), and also used to detect phytoplasma and spiroplasma infections in the salivary glands of infected insects (Markham and Alivizatos, 1983). DAPI (4'-6'-diamidino-2-phenylindole) specifically binds to adenine-thymine rich DNA of various origins (Russell *et al.*, 1975). DAPI staining was widely used to detect phytoplasmas in infected plant tissues (Seemüller, 1976; Hiruki, 1988a, b, c). The use of DAPI as a fluorochrome provided a highly sensitive technique for detection of phytoplasmas in sections from herbaceous plants (Hiruki and da Rocha, 1984, 1986), as well as tree species (Hiruki, 1981). Although staining is a simple and fast detection method for diagnosis of phytoplasma infection as well as for selecting disease-free stock

materials from a large number of samples suspected to be infected by phytoplasmas, it does not differentiate phytoplasmas since it is nonspecific. However, this is obviously less important when the sample is from experimentally infected insects or plants, where suitable controls are available, but not so suitable for assaying field-collected insects and plants.

I.4.3 Immunology

The application of immunological techniques has proven to be useful in analyzing the antigens of phytoplasma and for developing rapid disease diagnostic methods (Sinha, 1974; Caudwell *et al.*, 1983; Clark *et al.*, 1983; Sinha and Benhamou, 1983; Kirkpatrick and Garrott, 1984; Lin and Chen, 1985a, b, c, 1986; Hobbs *et al.*, 1987; Chen and Jiang, 1988; Clark *et al.*, 1989; Jiang *et al.*, 1989; Hsu *et al.*, 1990; Shen and Lin, 1993). Using partially purified phytoplasma preparations (intact organisms or membrane fractions) as immunogens, antisera and monoclonal antibodies have been raised against several phytoplasmas of aster yellows, clover phyllody, peach X-disease, primula yellows, western X-disease, grapevine flavescence doree, American tomato big bud, stolbur, maize bushy stunt, elm yellows, paulownia witches'-broom, ash yellows, and sweet potato witches'-broom (Chen and Jiang, 1988; Boudon-Padieu *et al.*, 1989; Clark *et al.*, 1989; Errampalli *et al.*, 1989; Jiang *et al.*, 1989; Schwartz *et al.*, 1989; Garnier *et al.*, 1990; Hsu *et al.*, 1990; Chang and Chen, 1991; Shen and Lin, 1993).

I.4.3.1 *Monoclonal Antibodies*

Polyclonal antisera often have relatively low specific titers and react with antigens from healthy plants or insect vectors, although antisera prepared from phytoplasmas purified from insects have less background reaction with healthy plant antigens (Chen and Jiang, 1990). Therefore, polyclonal antisera are not useful for differentiating phytoplasmas (Lee and Davis, 1992). Monoclonal antibodies produced by hybridoma techniques are monospecific, each reacting with only one epitope of the selected antigen. The high specificity and sensitivity of monoclonal antibodies have greatly improved the reliability of immuno-identification techniques. Phytoplasma antigens were detected in preparations from diseased lettuce and periwinkle plants using monoclonal antibodies against yellows agent (Lin and Chen, 1985a, b, 1986; Jiang *et al.*, 1988). These monoclonal antibodies did not react with phytoplasmas from ash yellows, elm yellows, maize bushy stunt, paulownia witches'-broom, and peanut rosette infected plants. Monoclonal antibodies are particularly useful for differentiating closely related strains of phytoplasmas (Lin and Chen, 1985a, b, 1986; Chen and Jiang, 1988; Clark *et al.*, 1989; Jiang *et al.*, 1989; Sears *et al.*, 1989; Garnier *et al.*, 1990; Guo *et al.*, 1991; Shen and Lin, 1993). The disadvantage of monoclonal antibodies is in their highly monospecific recognition of a single epitope, i.e. homogeneous monoclonal antibody recognizes only a single antigenic site (epitope) on complex immunogens. Therefore, if a particular epitope is shared among different immunogens, it is unable to differentiate the immunogens. Thus, certain monoclonal antibodies may sometimes fail in diagnosing plants showing disease symptoms associated with phytoplasmas (Lin and Chen, 1986; Hiruki, 1988b).

I.4.3.2 *Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISA developed for detection of viral antigens in tissue sections has reached a sensitivity comparable with that of radio-immunoassay (Nakane and Pierce, 1966; Engvall and Perlmann, 1971). Extremely high sensitivity of antigen-detectability has been reported by incorporating the biotin/avidin system into ELISA (Kendall *et al.*, 1983; Hahn *et al.*, 1986). ELISA is very useful for screening a large number of samples (Clark *et al.*, 1978). It has been widely used in field surveys of plant viruses and spiroplasmas. ELISA has been employed to assay uncultured phytoplasmas (Sinha and Benhamou, 1983; Jiang *et al.*, 1988). Earlier application, using polyclonal antisera prepared from partially purified phytoplasmas in ELISA for identification of phytoplasmas in infected plants, indicated that polyclonal antisera could not readily differentiate some related phytoplasma strains (Sinha and Benhamou, 1983; Sinha and Chiykowski, 1984). However, with proper controls, which have to be included in the assays, such as cross-absorbing antisera with healthy plant extracts, polyclonal antisera clearly detected disease-specific-antigens in infected plants and, in most cases, effectively differentiated distantly related phytoplasmas (Sinha, 1988; Clark *et al.*, 1989; Errampalli *et al.*, 1989). The combination of ELISA and immunofluoresence microscopy using monoclonal antibodies greatly improved both the specificity and sensitivity of ELISA for detection and differentiation of phytoplasmas (Lin and Chen, 1985b; Clark *et al.*, 1989). Monoclonal antibodies raised against primula yellows cross-reacted with the European strain of aster yellows and differentiated aster yellows from clover phyllody phytoplasmas (Clark *et al.*, 1989). ELISA using phytoplasma-specific monoclonal antibodies has also

been applied to detection of phytoplasmas in individual insect vectors (Boudon-Padieu *et al.*, 1989)

I.4.3.3 Dot Blot Immunoassay

A dot blot immunoassay has been used for detection of spiroplasmas (Chen *et al.*, 1989) and was recently extended to the diagnosis of phytoplasma infection (Boudon-Padieu *et al.*, 1989; Hsu *et al.*, 1990). The sensitivity of dot blot immunoassays was about 1.5 orders of magnitude greater than that of indirect ELISA (Hsu *et al.*, 1990). A simplified blotting procedure, tissue blotting, in which a freshly cut tissue surface is pressed directly on nitrocellulose membranes, was developed and applied for detection of plant viruses and phytoplasmas (Lin *et al.*, 1990). Examination of immunologically stained tissue blots of infected leaf tissue (cross sections) revealed that phytoplasma antigens in leaves were restricted to the phloem cells of midrib and secondary veins. Dot blot immunoassays using tomato big bud phytoplasma-specific monoclonal antibodies were used recently to differentiate phytoplasma strains in the aster yellows (AY) phytoplasma strain cluster (Lee *et al.*, 1990b). The monoclonal antibodies specifically reacted with tomato big bud phytoplasma and with all other strains in the aster yellows phytoplasma strain cluster, while no other phytoplasmas reacted. The dot blot immunoassay was also applied for detecting phytoplasmas in insect vectors (Garnier, 1990).

I.4.3.4 *Immunofluorescence Microscopy*

Indirect immunofluorescence staining, usually in conjugation with the ELISA, has been widely used to detect phytoplasmas in tissues (Lin and Chen, 1986; Lherminier *et al.*, 1989; Caudwell *et al.*, 1990; Sinha and Chiykowski, 1990). Although polyclonal antisera could be used in this approach for detection of phytoplasmas in infected tissues for identification and differentiation of phytoplasmas, they did not show high specificity for the phytoplasma against which they had been prepared (da Rocha *et al.*, 1986). In contrast, monoclonal antibodies bound specifically to phytoplasmas in sections of sieve tubes of diseased plants (Lin and Chen, 1986). The advantages of immunofluorescence microscopy are high sensitivity, specificity, and simplicity. Immuno-fluorescence microscopy may be useful for revealing the intracellular distribution of phytoplasmas at the light microscopy level (Hiruki, 1988a).

I.4.4 Molecular biology

The recent development of recombinant DNA and hybridoma techniques have allowed significant progress to be made in detection and identification of phytoplasmas. Since the first DNA probes cloned from the insect vector *Colladonas montanus* were reported by Kirkpatrick *et al.* (1987), other DNA fragments have been cloned from at least 20 phytoplasma strains: Western X-disease (Kirkpatrick *et al.*, 1987; Lee *et al.*, 1991b), Maryland aster yellows (Lee *et al.*, 1988a), maize bushy stunt (Davis *et al.*, 1988), *Oenothera* virescence (Sears *et al.*, 1989), apple proliferation (Kollar *et al.*, 1990), periwinkle little leaf 0-1 (Davis *et al.*, 1990a), elm yellows (Lee *et al.*, 1988a), tomato big bud (Lee *et al.*, 1988a, 1990c), clover proliferation (Deng and Hiruki, 1990b), ash

yellows (Davis *et al.*, 1991), beet leafhopper-transmitted virescence (Lee *et al.*, 1990b; Shaw *et al.*, 1990), Canada peach X-disease (Lee *et al.*, 1991b), walnut witches'-broom (Chen and Chang, 1991; Chen *et al.*, 1992), pigeon pea witches'-broom (Harrison *et al.*, 1991), lethal yellowing of palm (Harrison *et al.*, 1992), rice yellow dwarf (Nakashima *et al.*, 1991), grapevine flavescence doree (Davis *et al.*, 1992; Guo *et al.*, 1992), Italian periwinkle virescence (Davis *et al.*, 1992), and blueberry stunt (Tang and Chen, 1992). Using cloned phytoplasma DNA fragments as molecular probes in DNA-DNA hybridization provides sensitive and specific detection of phytoplasmas in infected plant or insect tissues (Kirkpatrick *et al.*, 1987, 1989; Davis *et al.*, 1988, 1990b, 1991; Lee and Davis 1988; Lee *et al.*, 1988b, 1990a; Deng and Hiruki, 1990b, c). Following the first successful application of the polymerase chain reaction (PCR) in phytoplasma study (Deng and Hiruki, 1990a) provided the most sensitive detection of phytoplasmas, numerous papers have reported the detection and identification of phytoplasmas by PCR (Ahrens and Seemüller, 1992; Schaff *et al.*, 1992; Lee *et al.*, 1993a, b, 1994; Davis and Lee, 1993; Schneider *et al.*, 1993; Gundersen *et al.*, 1994a, b, 1996). The combination of PCR with other molecular biological techniques such as restriction length fragment polymorphism (RFLP), heteroduplex mobility assay (HMA), DNA sequencing etc. permits the establishment of a genetically based phytoplasma classification.

I.4.4.1 *Nucleic Acid Hybridization*

Cloned phytoplasma DNA or their complementary RNA (Lee *et al.*, 1988b) labeled with ^{32}P nucleotides or nonradioactive biotin (Deng and Hiruki, 1990d) used as probes have been widely applied in dot-blot and Southern-blot hybridization assays for

detection and identification of phytoplasmas in plants and insect hosts (Kirkpatrick *et al.*, 1987, 1990; Davis *et al.*, 1988, 1990a, b, 1991; Lee *et al.*, 1988a, b, 1991a, b; Sears *et al.*, 1989; Bertaccini *et al.*, 1990a, b; Bonnet *et al.*, 1990; Kollar *et al.*, 1990; Deng and Hiruki, 1990c, 1991b; Chen and Chang, 1991; Hibben *et al.*, 1991; Nakashima *et al.*, 1991; Chen *et al.*, 1992; Shaw *et al.* 1993). For example, a phytoplasma was detected by an extrachromosomal DNA probe of walnut witches'-broom (WWB) phytoplasma that hybridized to DNA from WWB and pecan bunch tissues, but not to DNA from other unrelated phytoplasmas such as western X, eastern aster yellows, western severe aster yellows, beet leafhopper-transmitted virescence agent, and *Spiroplasma citri* (Chen *et al.*, 1992). A number of probes derived from various phytoplasmas were also used to investigate their genetic inter-relatedness. Clover proliferation and potato witches'-broom phytoplasmas were shown to be closely related to each other, but distinct from aster yellows and clover phyllody phytoplasmas on the basis of dot-blot and Southern-blot hybridization (Deng and Hiruki, 1990b, c, d, 1991a, b). Hybridization studies have shown that phytoplasmas from diverse geographic origins share high nucleotide sequence homology with one another but show relatively little homology with other phytoplasmas or culturable mollicutes (Davis *et al.*, 1988, 1990a, b; Lee and Davis, 1988, Lee *et al.*, 1988b, 1990a; Nakashima *et al.*, 1993). Several distinct genomic strain clusters such as aster yellows, elm yellows, peach X, and virescence etc. were established on the basis of dot-blot and Southern-blot hybridization/RFLP analyses (Lee and Davis, 1988; Lee *et al.*, 1988a, 1990b, 1991b; Kuske *et al.*, 1991a, b). As more cloned phytoplasma DNA probes from various sources become available for DNA hybridization analyses, genetic characteristics rather than biological properties such as insect transmission characteristics

and phenotypic or pathological characteristics exhibited by infected hosts, will provide a more reliable basis for phytoplasma classification.

I.4.4.2 *Polymerase Chain Reaction (PCR)*

Polymerase chain reaction (PCR), first expounded in a paper by Kleppe *et al.* (1971) and developed into a technique in the mid-1980s (Mullis *et al.*, 1986; Saiki *et al.*, 1985, 1986), provides a highly sensitive method for detection of DNA. It enables the copying of a single DNA molecule over a billion times in a few hours. The number of applications of PCR has been rising at an exponential rate like the PCR reaction itself. Recently, the PCR technique has been widely applied for detection and classification of phytoplasmas. Phytoplasma DNA can be detected in amounts as little as about 1 to 6×10^{-2} pg total nucleic acids from periwinkle infected with clover proliferation and grapevine yellows (Deng and Hiruki, 1990a; Chen *et al.*, 1993) and 5 to 170 pg total nucleic acid from infected host plants (Ahrens and Seemüller, 1992; Schaff *et al.*, 1992; Wang *et al.*, 1994). The detection of phytoplasmas by PCR is at least 100 to 1,000,000 times as sensitive as that by dot-blot hybridization methods (Deng, 1991; Chen *et al.*, 1993)). PCR assays using universal primer pairs designed on the basis of 16S rDNA sequence have been employed effectively to detect and identify a broad array of known and unknown phytoplasmas from various host plants and insect vectors (Deng and Hiruki, 1991a; Ahrens and Seemüller, 1992; Lee *et al.*, 1993b; Namba *et al.*, 1993a; Schnieder *et al.*, 1993; Wang *et al.*, 1994; Pollini *et al.*, 1996). However, universal primer pairs alone are not applicable for epidemiological studies where more than one type of phytoplasmas are associated with a given disease (Ceranic-Zagorac and Hiruki, 1996). Group specific

primers have been applied to detect mixed-phytoplasma infections in a single host plant (Deng and Hiruki 1990a; Lee *et al.*, 1994; Namba *et al.*, 1993b). A modified PCR, named recycled PCR, was also developed to enable detection of mollicute-specific DNA fragments and phytoplasma-specific or group-specific DNA fragments as multiple bands (Namba *et al.*, 1993b). Because of relatively low titers (Kollar *et al.*, 1990) and an uneven distribution of phytoplasmas in plant hosts, single amplification sometimes failed to detect phytoplasmas from woody hosts such as fruit and ornamental tree species. Nested-PCR assays provided higher specificity and increased over 100 fold detection sensitivity allowing ready detection of phytoplasmas from all tested infected woody hosts and insect vectors (Gundersen and Lee, 1996). Furthermore, amplification of nonspecific fragments from healthy plants may occur by using primers for the 16S rRNA gene (Deng and Hiruki, 1991a; Ahrens and Seemüller, 1992; Yoshikawa *et al.*, 1994; Nakamura *et al.*, 1996). Use of primers designed on the basis of nucleotide sequences of ribosomal protein genes did not amplify nonspecific fragments and provided more reliable detection of phytoplasmas (Yoshikawa *et al.*, 1994; Nakamura *et al.*, 1996). In summary, the detection sensitivity of phytoplasmas has been shown in the order of nested-PCR > direct PCR > RNA riboprobes > dsDNA probes > oligonucleotide probes >monoclonal antibodies > polyclonal antisera > DNA staining. It is obvious that PCR has provided the most sensitive detection of phytoplasmas.

I.4.4.3 *Restriction Length Fragment Polymorphism (RFLP)*

Earlier application of RFLP for differentiation and classification of phytoplasmas was combined with dot or Southern hybridization analyses. The very similar genomic

organization between PWB phytoplasma and CP phytoplasma was identified by dot hybridization and RFLP analyses (Deng and Hiruki, 1991b; Lee *et al.*, 1991a). A comparative study of grapevine flavescence doree and southern European grapevine yellows by RFLP and biotinylated DNA probes revealed that they shared some regions of DNA sequence homology but were distinct from each other (Davis *et al.*, 1992). Fifteen phytoplasma isolates from North America and Europe also were studied by RFLP analyses and were classified into three distinct genotypic groups (Lee *et al.*, 1991a). Since the introduction of PCR into phytoplasma studies, RFLP analysis of PCR-amplified 16S rDNA has been widely applied for identification and classification of a broad range of phytoplasmas (Lee *et al.*, 1993b; Namba *et al.*, 1993b; Schneider *et al.*, 1993; Seemüller *et al.*, 1994; Gundersen *et al.*, 1994a, b, 1996; Ceranic-Zagorac and Hiruki, 1996). Eleven distinct phytoplasma 16S rDNA groups and more than twenty-five subgroups have been identified by using this approach (Lee *et al.*, 1993b; Gunderson *et al.*, 1994b, 1996). However, the closely related phytoplasmas could not be differentiated by analyses of 16S rDNA fragment because of their highly conserved nature (Lee *et al.*, 1992b, 1993b; Griffiths *et al.*, 1994a). Ribosomal protein genes had a greater potential to reveal variation among closely related strains (Gunderson *et al.*, 1996). Further studies on the RFLP profiles of phytoplasma ribosomal protein gene sequences amplified by PCR will reveal a finer level of strain differentiation within each phytoplasma group than 16S rDNA analyses revealed.

I.4.4.4 *Heteroduplex Mobility Assay (HMA)*

Heteroduplex mobility assay (HMA), first developed for the detection and estimation of genetic divergence between human immunodeficiency virus (HIV) strains (Delwart *et al.*, 1993), is a new, rapid, accurate and simple screening tool which is not only capable of distinguishing between individual strains but can also provide reliable inferences regarding phylogenetic relationships among strains of microorganisms. HMA is based on the observation that DNA heteroduplexes formed between different single nucleotide strands have a reduced mobility in polyacrylamide gels under nondenaturing conditions, and their mobilities are proportional to their degree of divergence. Heteroduplexes are generated by base paring between complementary single strands derived from different parental duplex molecules during genetic recombination. Unknown DNA sequences can be compared against themselves or standard reference sequences. The DNA sequence of genetically common or rare variants could, therefore, be determined on a selective rather than random basis. In addition, heteroduplex analysis can be used for tracking specific sequence variants within individual samples (Delwart *et al.*, 1994) assisting in establishing epidemiological linkages between individual samples. Recently, HMA has been employed to detect and to differentiate phytoplasmas (Zhong and Hiruki, 1994; Ceranic-Zagorac and Hiruki, 1996; Cousin *et al.*, 1997). All results for differentiation of phytoplasmas from HMA were in agreement with those obtained by PCR and in particular RFLP analyses. The second step of RFLP after PCR amplification for classification of phytoplasmas can be avoided by HMA. It has been demonstrated that HMA provided sensitive differentiation of phytoplasmas when other methods such as RFLP were not readily applicable to differentiate between very closely related

phytoplasmas (Ceranic-Zagorac and Hiruki, 1996; Cousin *et al.*, 1997). Obviously, HMA combined with PCR will be a very simple, fast, sensitive and reliable method for detection and classification of different strains and groups of phytoplasmas.

I.5 ETIOLOGY OF PHYTOPLASMA DISEASES

Historically, the etiology of phytoplasma diseases has been studied mainly by observation of phytoplasmas in plant phloem tissues by electron microscopy and has not made a significant progress until the recent development of molecular probes for DNA hybridization and serological assays due to the inability to culture these pathogens *in vitro*. The identity and/or relationships of phytoplasmas that cause several "yellows-type" diseases have been established by a variety of serological and nucleic acid-based assays. RFLP analyses of PCR amplified phytoplasmal 16S rDNA were widely used to investigate the etiology of grapevine yellows (Daire *et al.*, 1992, 1993a; Davis *et al.*, 1992, 1993; Prince *et al.*, 1993; Alma *et al.*, 1996) The results of these studies indicated that grapevine yellows diseases were induced by a variety of genetically diverse phytoplasmas. Grapevine *flavescence doree* (FD) phytoplasma was shown to be a member of the elm yellows group, while other grape isolates were similar to stolbur (Daire *et al.*, 1993b). The phytoplasmas associated with grapevine yellows from Italy, Germany, France, and United States were divided into three distinct groups: the FD-phytoplasma was related to the elm yellows group (16SrV) and several periwinkle isolates were similar to AY group (16SrI-B and 16SrI-G), while a Virgina yellows agent was very similar to the X-disease group (16SrIIIA) (Daire *et al.*, 1992; Prince *et al.*, 1993;

Alma *et al.*, 1996). Using monoclonal antibodies, cloned phytoplasma chromosomal DNA probes and a PCR system, it was found that phytoplasmas associated with GY in New York were related to some Italian strains, and that periwinkle-maintained isolates were somewhat different from phytoplasmas in grapevines (Chen *et al.*,1993). RFLP analysis using cloned probes from AP and vaccinium witches'-broom (VAC) phytoplasma and a 16S rDNA probe from X-disease phytoplasma showed that most European stone fruit diseases were caused by AY phytoplasmas related to AP, whereas VAC was related to X-phytoplasmas. No X-phytoplasma type stone fruit diseases were found in Europe (Ahrens *et al.*, 1993). Cloned DNA probes derived from a periwinkle-maintained isolate of elm yellows (EY) showed the EY-phytoplasma was genetically distinct from other phytoplasmas and the EY isolates from the United States were similar to those in Europe (Lee *et al.*, 1993a). Using cloned probes from an European elm witches'-broom (ULW) as probes in Southern blots, a close relationship was shown among European elm witches'-broom, North American elm yellows, and European alder yellows. (Mäurer *et al.*, 1993). By means of monoclonal antibodies and cloned DNA probes, the ash yellows (AshY) phytoplasmas in USA and lilac phytoplasmas were shown to be similar (Hibben *et al.*, 1991). The host range of AshY phytoplasmas in nature includes 12 ash species and 19 lilac species (Sinclair *et al.*, 1996). Monoclonal antibodies against the sweet potato witches'-broom (SPWB) phytoplasma cross-reacted with phytoplasmas associated with peanut and asparagus bean witches'-broom but not with loofah, paulownia, and *Ipomoea obscura* witches'-broom, aster and elm yellows, and rice yellow dwarf (Shen and Lin, 1993)

Direct and nested-PCR assays using universal, group- and pathogen-specific primers and RFLP analysis of phytoplasmas associated with spartium witches'-broom revealed two different agents present in plants with similar symptoms. The prevalent agent was close to the AP strain cluster whereas the less frequently detected phytoplasma was similar to EY phytoplasma (Marcone *et al.*, 1996). A similar approach was used to study papaya diseases such as dieback, mosaic, and yellow crinkle. A close relationship was found between phytoplasmas associated with yellow crinkle and mosaic but they differed from the phytoplasma of dieback disease. Papaya yellow crinkle and mosaic, as well as sweet potato little leaf and crotalaria witches'-broom phytoplasmas are genetically related to the western X-phytoplasma while papaya dieback phytoplasma is related to stolbur phytoplasma that is a member of the aster yellows phytoplasma cluster (Gibb *et al.*, 1996). Hybridization studies have shown that phytoplasmas from diverse geographic origins share high nucleotide sequence homology with one another (Davis *et al.*, 1988, 1990a, b; Lee and Davis, 1988, Lee *et al.*, 1988b, 1990a; Nakashima *et al.*, 1993). Namba et al. (1993b) found that all Japanese group I phytoplasmas including paulownia witches'-broom, tomato yellows, onion yellows and mulberry dwarf share the same 16S rDNA sequences. Paulownia and jujube witches'-broom are very serious diseases and prevalent in China, Japan, and Korea. However, there is little research concerning the inter- and intra-relatedness of their pathogens to date. This is not only a major obstacle in developing diagnostic procedures, epidemiological studies, and formulating control measures of these diseases, but also has a serious negative impact on quarantine regulations.

I.6 TAXONOMY OF PHYTOPLASMAS

Since the discovery of phytoplasmas that were first termed MLOs because of their resemblance in morphology and ultrastructure to animal mycoplasmas (members of the class *Mollicutes*) (Doi *et al.*, 1967), the taxonomic status of phytoplasmas has been uncertain due to the current inability to culture them *in vitro*. The members of the class *Mollicutes* are characterized by the lack of a cell wall, small genome sizes (680 - 1600 kb), low guanine-plus cytosine (G+C) content, and unusual nutritional requirements (Woese *et al.*, 1980; Rogers *et al.*, 1985; Weisburg *et al.*, 1989; Razin, 1992). On the basis of analyses of 16S rRNA and 5S rRNA gene sequences, it has been postulated that the *Mollicutes* arose by degenerative evolution from gram-positive clostridium-like ancestors of a lactobacillus lineage (Woese *et al.*, 1980, 1987; Weisburg *et al.*, 1989; Maniloff, 1992). Phytoplasmas have many characteristics in common with culturable members of the class *Mollicutes* (Sears and Kirkpatrick, 1994). Comparisons of 16S rRNA or ribosomal protein (rp) gene sequences of some representative phytoplasma strains and *Acholeplasma laidlawii* revealed that phytoplasmas are related to *Acholeplasma*, and are evolutionarily distinct from animal mycoplasmas (Lim and Sears, 1989, 1992; Kuske and Kirpatrick, 1992; Kirkpatrick *et al.*, 1992). At least four types of molecular data support the placement of phytoplasmas in the class Mollicutes (Sears and Kirkpatrick, 1994): 1) the G+C genomic content of phytoplasmas (25 to 30%) resembles the A+T-rich DNA values of culturable members of *Mollicutes*. 2) the genetic phylogeny based on sequences from 16S rRNA genes of several phytoplasmas places them on the Acholeplasma-Anaeroplasma branch within the *Mollicutes*. 3) because phytoplasma

genome sizes vary from 450 to 1,180 kb, the genome complexity is in the same range as that of many *Mycoplasma* species, but the genome is substantially smaller than that of their phylogenetic cousins, the acholeplasmas and anaeroplasmas. 4) sequence data from several ribosomal protein genes confirm the phytoplasma-*Acholeplasma* phylogenetic relationship.

In classical plant mollicute studies, pathogen identification and classification have relied primarily on qualitative data such as symptomatology, host range, and vector specificity (McCoy *et al.*, 1989). However, classifying prokaryotes solely on the basis of a few nutritional or morphological characteristics is complicated and laborious and often has resulted in misleading conclusions because the phytoplasma-induced biological properties alone are less reliable than molecular data and are not sufficient for identification. Recently, molecular-based analyses have been used increasingly to identify and differentiate uncultured phytoplasmas. The development of molecular probes, such as phytoplasma-specific cloned DNA probes and monoclonal antibodies, has significantly improved phytoplasma identification and made it possible to classify phytoplasmas on the basis of DNA-DNA homology and serological data. (Davis *et al.*, 1988, 1991; Bonnet *et al.*, 1990; Deng and Hiruki, 1991b; Lee *et al.*, 1991a, 1992a, b, 1993a; Chen *et al.*, 1992; Harrison *et al.*, 1992; Nakashima *et al.*, 1993; Griffiths *et al.*, 1994a, b; Ko and Lin, 1994). The introduction of RFLP analysis of PCR amplified phytoplasmal conserved 16S rRNA gene sequences has greatly improved the ability of researchers to accurately identify and classify a broad range of phytoplasmas (Lim and Sears, 1989; Deng and Hiruki, 1991a, b; Ahrens and Seemüller, 1992; Davis and Lee, 1993; Firrao *et al.*, 1993; Namba *et al.*, 1993a; Schneider *et al.*, 1993; Vega *et al.*, 1993; Lee *et al.*, 1993b, 1994;

Griffiths *et al.*, 1994a, b; Gundersen *et al.*, 1994a, b; Vibio *et al.*, 1994; Gauthier *et al.*, 1995). The first work using this approach was carried out to differentiate 17 phytoplasma isolates into four distinct groups by Ahrens and Seemüller (1992). A similar RFLP analysis of PCR-amplified phytoplasmal 16S rRNA genes established nine distinct 16S ribosomal RNA (16Sr) groups and fourteen subgroups (Lee *et al.*, 1993b). Analysis of the AY cluster was investigated further by RFLP analysis using cloned chromosomal DNA probes in Southern blot hybridizations (Davis and Lee, 1993; Lee *et al.*, 1992a). Namba *et al.* (1993b) used specific primers to amplify the 16S rRNA genes of six Asian phytoplasmas and established three phylogenetic groups. Up to now, at least 55 phytoplasma strains have been examined by a variety of molecular techniques, including full-length 16S rDNA sequence analysis, specific 16S-23S rDNA spacer sequences, and PCR amplification with specific primers (IOM 1995). Eleven distinct phytoplasma 16S rDNA groups and more than 25 subgroups have been identified on the basis of the results of RFLP analysis of PCR-amplified 16S rDNA (Lee *et al.*, 1993b; Gundersen *et al.*, 1994b, 1996). Phylogenetic tree was constructed by parsimony analysis of 16S rRNA genes and ribosomal protein genes (Fig. I.1) (Gundersen *et al.* 1994). The identified phytoplasmas and plant diseases they caused are listed in Table I.1 (modified from Sinclair *et al.*, 1996). In this thesis the group numbers initiated by Lee *et al.* (1993b, Table I.1) for phytoplasma classification will be used.

I.7 CONTROL OF PHYTOPLASMA ASSOCIATED DISEASES

The ultimate goal in studying plant diseases is to prevent or control the diseases. However, effective control of plant diseases associated with phytoplasmas becomes very difficult because of insufficient knowledge of phytoplasma characteristics such as host range, insect vectors, multiplication, infection mechanism, and epidemiology etc. Since the discovery of association of phytoplasmas with certain yellows diseases (Doi *et al.*, 1967), various control measures have been used with varying degree of success. These include planting clean (disease-free) stocks, breeding of disease-resistant varieties, exclusion of insect vectors and inoculum sources, and chemotherapy.

Control of insect vectors and alternate plant hosts has been successfully used against certain fungal, bacterial and virus diseases. Although phytoplasmas are mainly transmitted by insect vectors and weed plant hosts act as sources of inoculum, control of insect vectors and weed plant hosts has been seldom successful in control of phytoplasma diseases (Thompson *et al.*, 1973), Investigation of potential insect vectors and their migration patterns may be of use to predict new disease outbreaks and disease spread, thus contributing to the effective management of these diseases.

Shoot tip culture (Heintz, 1989), callus culture (Moellers and Sarkar, 1989), and meristem tissue culture (Green *et al.*, 1989, Heintz, 1989), sometimes in combination with either heat or antibiotic treatment (Heintz, 1989) have been used for eliminating phytoplasmas from plants. Phytoplasmas are generally heat labile at temperature as low as 37°C and are not present in plant meristems after extended heat treatment (Caudwell, *et al.*, 1990). The critical step using this approach is to ensure the absence of pathogens in

the treated plant materials. This was done traditionally by graft inoculation to sensitive indicator host plants or by selection based on rouguing, but they were labor intensive and time consuming. Therefore, a rapid and sensitive detection technique plays a key role in obtaining disease-free stock. If phytoplasma-free plants can be established, control of phytoplasma diseases may be expected by combining with insect vector control. Thus, establishment of clean stocks is the first and essential step toward controlling phytoplasma diseases at present.

Breeding of phytoplasma-resistant or tolerant varieties may be the most cost-efficient and useful way to protect plants from phytoplasma infection, although there are only few successful cases up to now, such as eggplant resistant to little leaf disease (Memane and Joi 1987), elm resistant to elm yellows (Mittempergher *et al.*, 1990), and coconut resistant to lethal yellowing (McCoy *et al.*, 1983). Genetic engineering have made it possible to introduce certain insect-resistant genes into plants (McCowan *et al.*, 1991; Strauss *et al.*, 1991; Boulter 1990, 1993; Tang *et al.*, 1994). These approaches, when successful, will promise control of phytoplasmal diseases in the future.

I.8 OBJECTIVES OF THIS STUDY

On the basis of the above mentioned problems in studying of phytoplasma diseases, this study was focused on the following objectives:

1. To establish specific and sensitive methods for detection and identification of phytoplasmas.

2. To find a simple, economic, effective and practical way for preservation of phytoplasma DNA in tissues.
3. To study the genetic relatedness of important phytoplasma isolates from different geographical locations using molecular techniques.
4. To identify a new phytoplasma-associated disease using molecular techniques.
5. To develop a procedure for establishing phytoplasma-free clones of witches'-broom-affected paulownia plants.

Table I.1. Phytoplasmal disease groups based on relatedness of pathogens (modified from Sinclair *et al.*, 1996)

Typical disease	Phylogenetic subclade and corresponding 16S rRNA group of pathogen		Number of members	Diseases with related phytoplasmas[c]
Aster yellows	i[a]	I[b]	>30	Apricot chlorotic leaf roll; bigbud and stolbur of nightshade, pepper, tomato; blueberry stunt; maize bushy stunt; chrysanthemum yellows; dogwood stunt; grapevine yellows; Moliere's disease of cherry; mulberry dwarf; onion yellows; witches'-broom of paulownia and *Ipomoea obscura*; peach decline; periwinkle little leaf; phyllody of clover, hydrangea, safflower; plum leptonecrosis; sandal spike; tomato yellows; virescence of columbine, *Diplotaxis*, evening primrose, hydrangea, larkspur, periwinkle, *Plantago*, primrose, rape
Apple proliferation	ii	X	5	European stone fruit yellows, black alder witches'-broom, pear decline, oak decline
Peanut witches'-broom	iii	II	3	Red bird cactus witches'-broom; sweet potato witches'-broom
X disease of *Prunus* spp.	iv	III	16	Almond brown line; blueberry witches'-broom; clover yellow edge; goldenrod yellows; grapevine yellows; milkweed yellows; peach yellow leafroll; peach yellows; pear decline; pecan bunch; spirea stunt; X diseases of cherry, peach, prune; tsuwabuki witches'-broom; walnut witches'-broom
Rice yellow dwarf	v	XI	3	Sugarcane whiteleaf, Bermudagrass whiteleaf
Pigeon pea witches'-broom	vi	IX	1	
Lethal yellowing of palms	vii	IV	2	"Lethal disease" of palms (in Africa)
Ash yellows	viii	VII	3	Lilac witches'-broom, eggplant little leaf
Potato witches'-broom	ix	VI	5	Clover proliferation, tomato bigbud, virescence of periwinkle, alfalfa witches'-broom
Elm yellows	x	V	5	Alder decline, flavescence doree of grapevine, hemp-dogbane yellows, *Rubus* stunt
Loofah wtiches'-broom	xi	VIII	6	
Lime witches'-broom[d]				

[a] Subclade numerals (lower case) are those used by Gundersen *et al.* (1994)
[b] Group numerals (upper case) are those used by Lee *et al.* (1993)
[c] Some diseases are listed with more than one group, because more than one kind of phytoplasma has been associated with them.
[d] The *Candidatus* name "*Candidatus* Phytoplasma aurantifolia" has been proposed for the phytoplasma associated with lime witches'-broom (Zreik *et al.*, 1995)

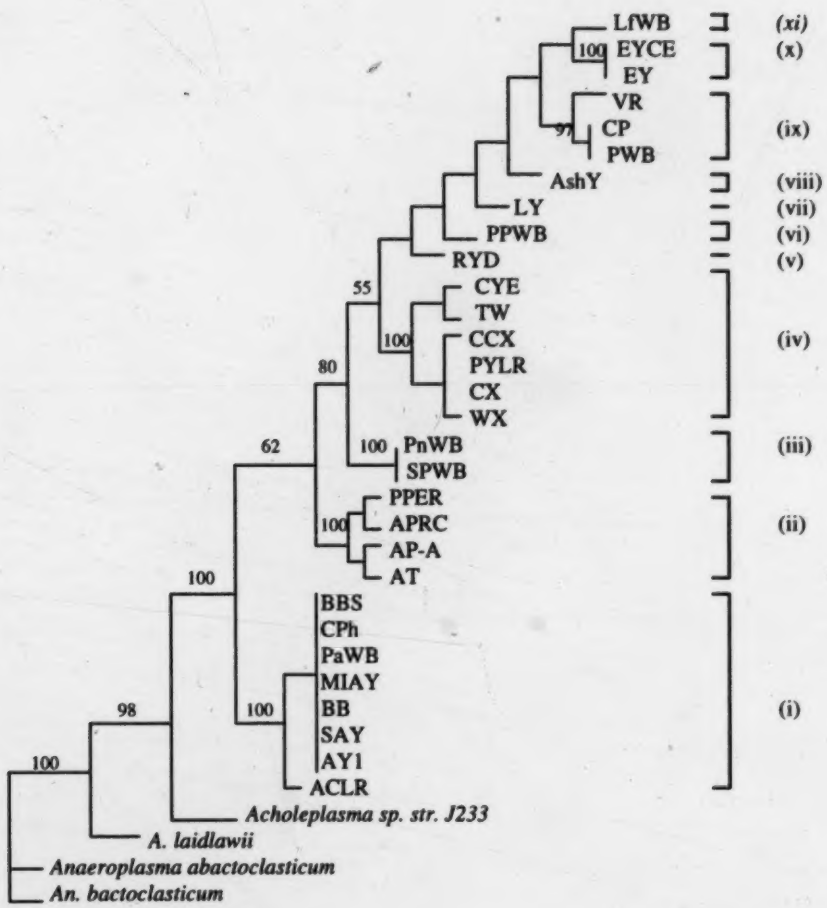


FIG. I.1. Phylogenetic tree constructed by parsimony analysis of 16S rDNA sequences from 30 phytoplasmas, two *Acholeplasma* spp., and two *Anaeroplasma* spp., employing *A. abactoclasicum* as the outgroup (Gundersen *et al.*, 1994).

1.8 References

Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82, 828-832.

Ahren U., Lorenz, K.H., and Seemüller, E. 1993. Genetic diversity among mycoplasmalike organisms associated with stone fruit diseases. Mol. Plant-microbe Interact. 6, 686-691.

Alma, A., Davis, R.E., Vibio, M., Danielli, A., Bosco, D., Arzone, A., and Bertaccini, A. 1996. Mixed infection of grapevines in northern Italy by phytoplasmas including 16S rRNA RFLP subgroup 16SrI-B strains previously unreported in this host. Plant Dis. 80, 418-421.

Banerjee, S.K., Makdisi, W.F., Weston, AP., Mitchell, S.M., and Campbell, D.R. 1995. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. Biotechniques 18, 768-773.

Bertaccini, A., Davis, R.E., and Lee, I.-M. 1990a. Distinctions among mycoplasmalike organisms (MLOs) in *Gladiolus*, *Ranunculus*, *Brassica*, and Hydrangea through detection with nonradioactive cloned DNA probes. Phytopathol. Medit. 29, 107-113.

Bertaccini, A., Davis, R.E., Lee, I.-M., Conti, M., Dally, E.L., and Douglas, S.M. 1990b. Detection of chrysanthemum yellows mycoplasmalike organism by dot hybridization and Southern blot analysis. Plant Dis. 74, 40-43.

Bonnet, F., Saillard, C., Kollar, A., Seemüller, E., Dosba, F., Bové J.M. 1990. Molecular probes for the apple proliferation MLO. Zentralbl. Bacteriol. Suppl. 20, 908-909.

Boudon-Padieu, E., Larrue, J., and Caudwell, A. 1989. ELISA and dot-blot detection of flavescence doree-MLO in individual leafhopper vectors during latency and inoculative state. Curr. Microbiol. 19, 357-364.

Boulter, D. 1993. Insect pest control by copying nature using genetically engineered crops. Phytochemistry 34, 1453-1466.

Boulter, D., Gatehouse, J.A., Gatehouse, A.M.R., and Hilder, V.A. 1990. Genetic engineering of plant for insect resistance. Endeaver, New Series 14, 185-190.

Caudwell, A., Boudon-Padieu, E., Lherminier, J., Schwartz, Y., and Meignoz, P. 1990. Current spread of the grapevine yellows and characterization methods for the MLO pathogen. Zentralbl. Bacteriol. Suppl. 20, 916-918.

Caudwell, A., Meignoz, R., Kuszala, C., Schneider, C., Larrue, J., Fleury, H., and Boudon, E. 1983. Serological purification and visualization in electron microscope of the grapevine flavescence doree (FD) pathogen (MLO) in diseased plants and infectious vector extracts. Yale J. Biol. Med. 56, 936-937.

Ceranic-Zagorac, P., and Hiruki, C. 1996. Comparative molecular studies on aster yellows phytoplasmas. Acta Horticulturae 432, 226-276.

Chang, C.J., and Chen, T.A. 1982. Spiroplasmas: cultivation in chemically defined medium. Science 215, 121-122.

Chang, C.J., and Chen, T.A. 1991. Utilization of monoclonal antibodies against elm yellows mycoplasmalike organisms in detection of elm yellows disease. Phytopathology 81, 121.

Chen, J., and Chang, C.J. 1991. Plasmid-like DNAs with a mycoplasma-like organism (MLO) and their seasonal occurrence. Phytopathology 81, 1169.

Chen, J., Chang, C.J., Jarret, R., and Gawel, N. 1992. Isolation and cloning of DNA fragments from mycoplasmalike organism associated with walnut witches'-broom disease. Phytopathology 82, 306-309.

Chen, K.H., Guo, J.R., Wu, X.Y., Loi, N., Carraro, L., Guo, Y.H., Chen, Y.D., Osler, R., Pearson, R., and Chen, T.A. 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows disease agent. Phytopathology 83, 915-922.

Chen, M.H., and Hiruki, C. 1977. Effect of dark treatment on the ultrastructure of aster yellows agent *in situ*. Phytopathology 67, 321-324.

Chen, T.A., and Jiang, X.F. 1988. Monoclonal antibodies against the maize bushy stunt agent. Can. J. Microbiol. 34, 6-11.

Chen, T.A., and Jiang, Y.P. 1990. Progress in the detection of plant mycoplasmalike organisms by using monoclonal and polyclonal antibodies. Zentralbl. Bakteriol. Suppl. 20, 270-275.

Chen, T.A., Lei, J.D., and Lin C.P. 1989. Detection and identification of plant and insect mollicutes. *In*: The Mycoplasmas, vol. 5. p. 393-424. R.F. Whitcomb and J.G. Tully (ed.), Academic Press, New York.

Chen, T.R. 1977. *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res. 104, 255-262.

Chiykowski, L.N. 1977. Cryopreservation of aster yellows agent in whole leafhoppers. Can. J. Microbiol. 23, 1038-1040.

Chiykowski, L.N. 1983. Frozen leafhoppers as a vehicle for long-term storage of different isolates of the aster yellows agent. Can. J. Plant Pathol. 5, 101-106.

Chiykowski, L.N. 1988. Maintenance of yellows-type mycoplasmalike organisms. *In*: Tree Mycoplasmas and Mycoplasma Diseases, p. 123-134. C. Hiruki (ed.), The University of Alberta press, Edmonton.

Chiykowski, L.N., and Sinha, R.C. 1982. Herbaceous host plants of peach eastern X-disease agent. Can. J. Plant Pathol. 4, 8-15.

Clark, M.F., Barbara, D.J., and Davis, D.L. 1983. Production and characteristics of antisera of *Spiroplasma citri* and clover phyllody-associated antigens derived from plants. Ann. Biol. 103, 251-259.

Clark, M.F., Flegg, C.L., Bar-Joseph, M., and Rottem, S. 1978. The detection of *Spiroplasma citri* by enzyme-linked immunosorbent assay (ELISA). Phytopathology 92, 332-337.

Clark, M.F., Morten, A., and Buss, S.L. 1989. Preparation of mycoplasma immunogens from plants and a comparison of polyclonal and monoclonal antibodies made against primula yellows MLO-associated antigens. Ann. Appl. Biol. 114, 111-124.

Cousin, M.T. Rous, J., Millet, N., and Michel, M.-F. 1990. Maintenance of MLOs (Mycoplasma-like organisms) on *Populus alba* micropropagation. J. Phytopathol. 130, 17-23.

Cousin, M.T., Roux, J., Boudon-Padieu, E., Bergés, R., Seemüller, E., and Hiruki, C. 1997. Use of heteroduplex mobility analysis (HMA) for differentiating phytoplasma isolates causing witches'-broom disease on *Populus nigra* cv Ltalica and stolbur or big bud symptoms on tomato. J. Phytopathol. (in press).

da Rocha, A., Ohki, S.T., and Hiruki, C. 1986. Detection of mycoplasmalike organisms *in situ* by indirect immunofluorescence microscopy. Phytopathology, 76, 864-868.

Daire, X., Boudon-Padieu, E., Berville, A., Schneider, B., and Caudwell, A. 1992. Cloned DNA probes for detection of grapevine flavescence doree mycoplasmalike organisms (MLO). Ann. Appl. Biol. 121, 95-103.

Daire, X., Clair, D., Larrue, J., Boudon-Padieu, E., and Caudwell, A. 1993a. Diversity among mycoplasma-like organisms inducing grapevine yellows in France. Vitis 32, 159-163.

Daire, X., Clair, D., Larrue, J., Boudon-Padiuu, E., Alma, A., Arzone, A., Carraro, L., Osler, R., Refatti, E., Granata, G., Credi, R., Tanne, E., Pearson, R., and Caudwell, A. 1993b. Occurrence of diverse MLOs in tissues of grapevine affected by grapevine yellows in different countries. Vitis 32, 247-248.

Davis, M.J., Konai, M., and Bak, W.-C. 1990. Electrophoretic separation of chromosomal DNA of mycoplasmalike organisms. IOM Letters, the 8th IOM Congress 1, 231-232.

Davis, M.J., Tsai, J.H., Cox, R.L., Mcdaniel, L.L., and Harrison, N.A. 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. Mol. Plant-Microbe Interact. 1, 295-302.

Davis, R.E., and Lee, I.-M. 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology 83, 1008-1011.

Davis, R.E., Dally, E.L., Bertaccini, A., Credi, R., Osler, R., Savino, V., Refatti, R., DiTerlizzi, B., Barba, M., and Lee, I.-M. 1992. RFLP analyses and dot hybridizations of chromosomal DNA distinguish two mycoplasmalike organisms (MLOs) associated with grapevine yellows disease. Phytopathology 82, 242.

Davis, R.E., Dally, E.L., Bertaccini, A., Lee, I.-M., Credi, R., Osler. R., Savino, V., Carraro, L., D. Terlizzi, B., and Barba, M. 1993. Restriction fragment length polymorphism analyses and dot hybridizations distinguish mycoplasmalike organisms associated with *flavescence doree* and southern European grapevine yellows disease in Italy. Phytopathology 83, 772-776.

Davis, R.E., Lee, I.-M., Douglas, S.M., and Dally E.L. 1990a. Molecular cloning and detection of chromosomal and extrachromosomal DNA of the mycoplasmalike organism (MLO) associated with little leaf disease in periwinkle (*Catharanthus roseus*). Phytopathology 80, 789-793.

Davis, R.E., Lee, I.-M., Douglas, S.M., Dally, E.L., and DeWitt, N.D. 1990b. Development and use of cloned nucleic acid hybridization probes for disease diagnosis and detection of sequence homologies among uncultured mycoplasmalike organisms (MLOs). Zentralbl. Bakteriol. Suppl. 20, 303-307.

Davis, R.E., Lee, I.-M., Douglas, S.M., Dally, E.L., and DeWitt, N.D. 1988. Cloned nucleic acid hybridization probes in detection and classification of mycoplasmalike organisms (MLOs). Acta Hortic. 234, 115-122.

Davis, R.E., Sinclair, W.A., Lee, I.-M., and Dally, E.L. 1991. Cloned DNA probes specific for detection of a mycoplasmalike organism associated with ash yellows. Mol. Plant-Microbe Interact. 5, 163-169.

Davis, R.E., Whitcomb, R.F., and Steere, R.L. 1968. Remission of aster yellows by antibiotics. Science 161, 793-795.

Del Giudice, R.A., and Hopps, H.E. 1978. Microbiological methods and fluorescent microscopy for direct demonstration of mycoplasma infection of cell cultures. *In*: Mycoplasma Infection of Cell Cultures, p. 57-59, G.J. McGarrity, D.G. Murphy and W.W. Nichols (ed.), Academic Press, New York.

Delwart, E.L., Sheppard, H.W., Walker, B.D., Goudsmit, J., and Mullins, J.I. 1994. Tracking HIV evolution *in vivo* using a DNA heteroduplex mobility assay. J. Virol. 68, 6672-6683.

Delwart, E.L., Shpaer, E.G., Louwagie, J., McCutchan, F.E., Grez, M., Rubsamen-Weigmann, H., and Mullins, J.I. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 *env* genes. Science 262, 1257-1261.

Denes, A.S., and Sinha, R.C. 1992. Alteration of clover phyllody mycoplasma DNA after in vitro culturing of phyllody-diseased clover. Can. J. Plant Pathol. 14, 189-196.

Deng, S. 1991. Molecular diagnosis and characterization of mycoplasmalike organisms associated with clover proliferation. Ph.D. Thesis. University of Alberta, Edmonton, Canada.

Deng, S., and Hiruki, C. 1990a. Enhanced detection of a plant pathogenic mycoplasmalike organism by polymerase chain reaction. Proc. Japan. Acad. 66B, 140-144.

Deng, S., and Hiruki, C. 1990b. Molecular cloning and detection of DNA of the mycoplasmalike organisms associated with clover proliferation. Can. J. Plant Pathol. 12, 383-388.

Deng, S., and Hiruki, C. 1990c. The use of cloned DNA probes for diagnosis of nonculturable plant mollicutes. Proc. Japan Acad. 66, 58-61.

Deng, S., and Hiruki, C. 1990d. Use of Biotinylated DNA probes for the detection and differentiation of mycoplasmalike organisms. Can. J. Plant Pathol. 12, 333.

Deng, S., and Hiruki, C. 1991a. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Methods 14, 53-61.

Deng, S., and Hiruki, C. 1991b. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81, 1475-1479.

Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or P.L.T. group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches'-broom, aster yellows or paulownia witches'-broom. Ann. Phytopathol. Soc. Jpn. 33, 259-266.

Engvall, E., and Perlmann, P. 1971. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol 109, 129-135.

Errampalli, D., Fletcher, J., and Sherwood, J.L. 1989. Production of monospecific polyclonal antibodies against the aster yellows mycoplasmalike organisms (AY MLO) of Oklahoma. Phytopathology 79, 1137.

Firrao, G., Gobbi, E., and Locci, R. 1993. Use of polymerase chain reaction to produce oligonucleotide probes for mycoplasmalike organisms. Phytopathology 83, 602-607.

Florence, E.R., and Cameron, H.R. 1978. Three-dimensional structure and morphology of mycoplasma-like bodies associated with albino disease of *Prunus avium*. Phytopathology 68, 75-80.

Garnier, M., Martin-Gros, G., Iskra, M., Zreik, L., Gandar, J., Fos, A., and Bové, J.M. 1990. Monoclonal antibodies against the MLOs associated with tomato stolbur and clover phyllody. Zentralbl. Bakteriol. Suppl. 20, 263-269.

Gauthier, G., Lafay, B., Ruimy, R., Breittmayer, V., Nicolas, J.L., Gauthier, M., and Christen, R. 1995. Small-subunit rRNA sequences and whole DNA relatedness concur for reassignment of *Pasteurella piscicida* (Snieszko et al.) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damsela* subsp. *Piscicida* comb. nov. Int. J. Syst. Bacteriol. 45, 139-144.

Gibb, K.S., Persley, D.M., Schneider, B., and Thomas, J.E. 1996. Phytoplasmas associated with papaya diseases in Australia. Plant Dis. 80, 174-178.

Goodwin, D., and Lee, S. 1993. Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. Biotechniques, 15, 438-444.

Green, S.K., Luo, C.Y., and Lee, D.R. 1989. Elimination of mycoplasmalike organisms from witches'-broom infected sweet potato. Phytopathol. Z. 126, 204-212.

Griffiths, H.M., Gundersen, D.E., Sinclair, W.A., Lee, I.-M., and Davis, R.E. 1994a. Mycoplasmalike organisms from milkweed, goldenrod, and spirea represent two new 16S rRNA subgroups and three new strain subclusters related to X-disease. Can. J. Plant Pathol. 16, 225-260.

Griffiths, H.M., Sinclair, W.A., Davis, R.E., Lee, I.-M., Dally, E.L., Guo, Y.-H., Chen, T.A., and Hibben, C.R. 1994b. Characterization of mycoplasmalike organisms from Fraxinus, Syringa, and associated plants from geographically diverse sites. Phytopathology 84, 119-126.

Gundersen D.E., and Lee, I.-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assay using two universal primer pairs. Phytopathol. Medit. 35, 144-151.

Gundersen, D.E., Lee, I.-M., Chang, C.J., and Davis, R.E. 1994a. RFLP analysis of ribosomal protein genes reveal strain diversity in MLO 16S rRNA groups I and III. Phytopathology 84, 1128.

Gundersen, D.E., Lee, I.-M., Rehner, S.A., Davis, R.E., and Kingsbury, D.T. 1994b. Phylogeny of mycoplasmalike organisms (phytoplasmas); a basis for their classification. J. Bacteriol. 176, 5244-5254.

Gundersen, D.E., Lee, I.-M., Schaff, D.A., Harrison, N.A., Chang, C.J., Davis, R.E., and Kingsbury, D.T. 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int. J. Syst. Bacteriol. 46, 64-75.

Guo, J.R., Chen, T.A., and Loi, N. 1991. Production of monoclonal antibodies against flavescence doree mycopalsmalike organism. Phytopathology 81, 1210.

Guo, J.R., Chen, T.A., Loi, N., and Pearson, R.C. 1992. Cloning of chromosomal DNA of the mycoplasmalike organism (MLO) associated with grapevine flavescence doree. Phytopathology 82, 243.

Hahn, I.F., Pickenhahn, P., Lenz, W., and Brandis, H. 1986. An avidin-biotin ELISA for the detection of staphylococcal enterotoxins A and B. J. Immunol. Meth. 92, 25-29.

Harrison, N.A., Bourne, C.M., Cox, R.L., Tsai, J.J., and Richardson, P.A. 1992. DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. Phytopathology 82, 216-224.

Harrison, N.A., Tsai, J.H., Bourne, C.M., and Richardson, P.A. 1991. Molecular cloning and detection of chromosomal and extrachromosomal DNA of mycoplasmalike organisms associated with witches'-broom disease of pigeon pea in Florida. Mol. Plant-Microbe Interact. 4, 300-307.

Heintz, W. 1989. Transmission of a new mycoplasmalike organism (MLO) from *Cuscuta odorata* (Ruiz. Et Pav.) to herbaceous plants and attempts to its elimination in the vector. Phytopathol. Z. 125, 171-186.

Hibben, C.R., Sinclair, W.A., Davis, R.E., and Alexander, J.H. III. 1991. Relatedness of mycoplasma-like organisms associated with ash yellows and lilac witches'-broom. Plant Dis. 75, 1227-1230.

Hiruki, C. 1981. Fluorescence microscopy in diagnosis of tree diseases associated with mycoplasma-like organisms (MLO). Proc. XII IUFRO World Con. Div. 2, 317-322.

Hiruki, C. 1988a. Fluorescence microscopy of yellows diseases associated with plant mycoplasmalike organisms. *In*: Mycoplasma Diseases of Crops: Basic and Applied Aspects, p. 51-76, K. Maramorosch and S.P. Raychaudhuri (ed.), Springer-Verlag, New York.

Hiruki, C. 1988b. Rapid and specific detection methods for plant mycoplasmas. *In*: Mycoplasma Diseases of Crops: Basic and Applied Aspects, p. 77-101, K. Maramorosch and S. P. Raychaudhuri (ed.), Springer-Verlag, New York Inc.

Hiruki, C. 1988c. Immunofluorescence microscopy of yellows diseases associated with plant mycoplasmalike organisms. *In*: Tree Mycoplasmas and Mycoplasma Diseases, p. 193-203, C. Hiruki (ed.) University of Alberta Press, Edmonton, Alberta.

Hiruki, C., and da Rocha, A. 1984. The use of fluorescent DNA binding agent, 4', 6'-diamidino-2-phenylindole (DAPI), for detecting mycoplasma infections in *Vinca rosea*. Can. J. Plant Pathol. 6, 262.

Hiruki, C., and da Rocha, A. 1986. Histochemical diagnosis of mycoplasma infections in Catharanthus roseus by means of fluorescent DNA binding agent, 4',6'-diamidino-2-phenylindole ·2HCl (DAPI). Can. J. Plant Pathol. 8, 185-188.

Hobbs, H.A., Reddy, D.V.R., and Reddy, A.H. 1987. Detection of a mycoplasma-like organism in peanut plants with witches'-broom using indirect enzyme-linked immunosorbent assay (ELISA). Plant Pathol. 36, 164-167.

Hsu, H.T., Lee, I.-M., Davis, R.E., and Wang, Y.C. 1990. Immunization for generation of hybridoma antibodies specifically reacting with plants infected with a

mycoplasmalike organism (MLO) and their use in detection of MLO antigens. Phytopathology 80, 946-950.

Hultuer L., and Cleaver E. 1994. Bacterial plasmid DNA miniprep using microwave lysis. Biotechniques, 16, 990-994.

Ishiie, T., Doi, Y., Yora, K., and Asuyama, H. 1967. Suppressive effects of antibiotics of tetracycline group on symptom development of mulberry dwarf disease. Ann. Phytopathol. Soc. Jpn. 33, 267-275.

Jacoli, G.G. 1974. Translocation of mycoplasma-like bodies through sieve pores in plant tissue cultures infected with aster yellows. Can. J. Bot. 52, 2085-2088.

Jacoli, G.G. 1978. Sequential degeneration of mycoplasma-like bodies in plant tissue cultures infected with aster yellows. Can. J. Bot. 56, 133-140.

Járausch, W., Lansac, M., and Dosba, F. 1996. Long-term maintenance of nonculturable apple-proliferation phytoplasmas in their micropropagated natural host plant. Plant Pathol. 45, 778-786.

Jensen, D.D. 1956. Insect transmission of virus between tree and herbaceous plants. Virology 2, 249-260.

Jiang, Y.P., Chen, T.A., Chiykowski, L.N., and Sinha, R.C. 1989. Production of monoclonal antibodies to peach eastern X-disease agent and their use in disease detection. Can. J. Plant Pathol. 11, 325-331.

Jiang, Y.P., Lei, J.D., and Chen, T.A. 1988. Purification of aster yellows agent from diseased lettuce using affinity chromatography. Phytopathology 78, 828-831.

Kendall, C., Ionescu-Matiu, I., and Dreesman, G.R. 1983. Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). J. Immunol. Meth. 56, 329-339.

Khadhair, A.-H., Momani, F., and Hiruki, C. 1995. Molecular stability of clover proliferation phytoplasma DNA in periwinkle plant tissues after thermal treatment under microwave conditions. Pro. Japan Acad. 71B, 265-268.

Kirkpatrick, B.C., and Fraser, J.D. 1989. Detection and differentiation of mycoplasmalike organisms using MLO-specific ribosomal RNA oligonucleotide sequences. Phytopathology 79, 1138.

Kirkpatrick, B.C., and Garrot, D.G. 1984. Detection of western X-disease mycoplasma-like organisms by enzyme-linked immunosorbent assay. Phytopathology 74, 825.

Kirkpatrick, B.C., Fisher, G.A., Fraser, J.D., and Purcell, A.H. 1990. Epidemiological and phylogenetic studies on western X-disease mycoplasmalike organisms. Zentralbl. Bakteriol. Suppl. 20, 288-297.

Kirkpatrick, B.C., Gao, J., and Harrison, N. 1992. Phylogenetic relationships of 15 MLOs established by PCR sequencing of variable regions within the 16S ribosomal RNA gene. Phytopathology 82, 1083.

Kirkpatrick, B.C., Strnger, D.C., Morris, T.J., and Purcell. A.H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasmalike organism. Science. 238, 197-200.

Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., and Khorana, H.G. 1971. Studies on polynucleotides: repair replication of short synthetic DNA's as catalyzed by DNA polymerases. J. Mol. Biol. 56, 341-361.

Ko, H.C., and Lin. C.P. 1994. Development and application of cloned DNA probes for a mycoplasmalike organism associated with sweetpotato witches'-broom. Phytopathology 84, 468-473.

Kollar, A., and Seemüller, E. 1989. Base composition of the DNA of mycoplasma-like organisms associated with various plant diseases. J. Phytopathol. 127, 177-186.

Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bové, J.M. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. Phytopathology 80, 233-237.

Kuske, C.R., and Kirkpatrick, B.C. 1989. Distribution and multiplication of western aster yellows MLOs in *Catharanthus roseus*. Phytopathology 80, 958.

Kuske, C.R., and Kirkpatrick, B.C. 1992. Phylogenetic relationships between the western aster yellows mycoplasmalike organisms and other prokaryotes established by 16S rRNA gene sequence. Int. J. Syst. Bacteriol. 42, 226-233.

Kuske, C.R., Kirkpatrick, B.C., and Seemüller, E. 1991a. Differentiation of virescence MLOs using western aster yellows mycoplasma-like organism chromosomal DNA probes and restriction fragment length polymorphism analysis. J. Gen. Microbiol. 137, 153-159.

Kuske, C.R., Kirkpatrick, B.C., Davis, J.M., and Seemüller, E. 1991b. DNA hybridization between western aster yellows mycoplasma-like organism plasmids and extrachromosomal DNA from other plant pathogenic mycoplasma-like organisms. Mol. Plant-Microbe Interact. 4, 75-80.

Lee, I.-M., and Davis, R.E. 1986. Prospect for *in vitro* culture of plant pathogenic mycoplasmalike organisms. Ann. Rev. Phytopathol. 24, 339-354.

Lee, I.-M., and Davis, R.E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1, 303-310.

Lee, I.-M., and Davis, R.E. 1992. Mycoplasmas which infect plants and insects. *In*: Mycoplasmas: Molecular Biology and Pathogenesis, p. 379-390. J. Manilaff, R.N. McElhaney, L.R. Rinch, and J.B. Baseman (ed.), American Society for Microbiology, Washington, D.C.

Lee, I.-M., Davis, R.E., and DeWitt, N.D. 1988a. Molecular cloning and screening by a new method for DNA fragments from elm yellows (EY) and tomato big bud (BB) mycoplasmalike organisms (MLOs). Phytopathology 78, 1602.

Lee, I.-M., Davis, R.E., and De Witt, N.D. 1990a. Nonradioactive screening method for isolation of disease-specific probes to diagnose plant disease caused by mycoplasmalike organisms. Appl. Environ. Microbiol. 56, 1471-1475.

Lee, I.-M., Davis, R.E., and Hiruki, C. 1990b. Beet leaf-hopper transmitted virescent and clover proliferation mycoplasmalike organisms (MLOs): two distinct strain types. Phytopathology 80, 958.

Lee, I.-M., Davis, R.E., and Hiruki, C. 1991a. Genetic relatedness among clover proliferation mycoplasmalike organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. Appl. Environ. Microbiol. 57, 3565-3569.

Lee, I.-M., Davis, R.E., and Hsu, H.T. 1990c. Monoclonal antibodies against tomato big bud mycoplasma-like organism (MLO) distinguish a group of interrelated MLO strains within the "aster yellows" strain cluster. Phytopathology 80, 958.

Lee, I.-M., Davis, R.E., Chen, T.A., Chiykowski, L.N., Fletcher, J., Hiruki, C., and Schaff, D.A. 1992a. A genotype-based system for identification and classification of mycoplasmalike organisms (MLOs) in the aster yellows MLO strain cluster. Phytopathology 82, 977-986.

Lee, I.-M., Davis, R.E., Hammond, R., and Kirkpatrick, B.C. 1988b. Cloned riboprobe for detection of a mycoplasmalike organism (MLO). Biochem. Biophys. Res. Commun. 155, 443-448.

Lee, I.-M., Davis, R.E., Sinclair, W.A., DeWitt, N.D., and Conti, M. 1993a. Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in USA and Italy

by means of DNA probes and polymerase chain reaction. Phytopathology 83, 829-833.

Lee, I.-M., Gundersen D.E., Davis, R.E., and Chiykowski, L.N. 1991b. Peach X-disease mycoplasmalike organism (MLO) genomic strain cluster. Phytopathology 81, 1169.

Lee, I.-M., Gundersen, D.E., Davis, R.E., and Chiykowski, L.N. 1992b. Identification and analysis of a genomic strain cluster of mycoplasmalike organisms associated with Canadian peach (eastern) X-disease, western X-disease, and clover yellow edge. J. Bacteriol. 174, 6694-6698.

Lee, I.-M., Gundersen, D.E., Hammond, R.W., and Davis R.E. 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology 84, 559-566.

Lee, I.-M., Hammond, R.W., Davis, R.E., and Gundersen, D.E. 1993b. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834-842.

Lherminier, J., Terwisscha Van Scheltinga, T., Boudon-Padieu, E., and Caudwell, A. 1989. Rapid immunofluorescent detection of the grapevine flavescence doree mycoplasmalike organism in the salivary glands of the leafhopper *Euscelidius variegatus* KBM. Phytopathol. Z. 125, 353-360.

Lim, P.O., and Sears, B.B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionarily distinct from animal mycoplasmas. J. Bacteriol. 171, 5901-5906.

Lim, P.O., and Sears, B.B. 1991. The genome size of a plant-pathogenic mycoplasmalike organism resembles those of animal mycoplasmas. J. Bacteriol. 173, 2128-2130.

Lim, P.-O., and Sears. B.B. 1992. Evolutionary relationships of a plant-pathogenic mycoplasmalike organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequence. J. Bacteriol. 174, 2606-2611.

Lin, C.P., and Chen, T.A. 1985a. Monoclonal antibodies against corn stunt spiroplasma. Can J. Microbiol. 31, 900-904.

Lin, C.P., and Chen, T.A. 1985b. Monoclonal antibodies against the aster yellows agent. Science 227, 1233-1235.

Lin, C.P., and Chen, T.A. 1985c. Production of monoclonal antibodies against *Spiroplasma citri*. Phytopathology 75, 848-851.

Lin, C.P., and Chen, T.A. 1986. Comparison of monoclonal antibodies and polyclonal antibodies in detection of the aster yellows mycoplasmalike organism. Phytopathology 76, 45-50.

Lin, N.S., Hsu, Y.H., and Hsu, H.T. 1990. Immunological detection of plant viruses and a mycoplasma-like organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80, 824-828.

Maia, E., and Beck, D. 1976. Culture d'organes *in vitro* et regeneration de plantes infected par des mycoplasmas. Ann. Phytopathol. 8, 303-306.

Maniloff, J. 1992. Phylogeny of mycoplasma. *In*: Mycoplasmas: Molecular Biology and Pathogenesis, p. 549-559, J. Maniloff, R. McElhaney, L. Finch, and J. Baseman (ed.), American Society for Microbiology, Washington, D.C.

Marcone, C., Ragozzino, A., Schneider, B., Lauer, U., Smart, C.D., and Seemüller, E. 1996. Genetic characterization and classification of two phytoplasmas associated with spartium witches'-broom disease. Plant Dis. 80, 365-371.

Markham, P.G., and Alivizatos, A.S. 1983. The transmission of corn stunt spiroplasma by natural and experimental vectors. *In*: Proc. Internat. Maize Virus Dis. Colloq. Workshop, p. 56-60. D.T. Gordon, J.K. Knoke, L.R. Nault, and R.M. Ritter (ed.), Ohio Agr. Res. Dev. Center, Wooster.

Mäurer, R., Seemüller, E., and Sinclair, W.A. 1993. Genetic relatedness of mycoplasmalike organisms affecting elm, alder, and ash in Europe and North America. Phytopathology 83, 971-976.

McCowan, B. H., McCabe, E.D., Russell, D.R., Robinson, D.J., Barton, K.A., and Raffa, K.F. 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Reports 9, 590-594.

McCoy, R.E. 1978. Attempts to culture mycoplasmalike agents at Fort Lauderdale. Proc. 3rd Int. Counc. Lethal Yellowing, 1977, Univ. Fla. Agr. Res. Rep. 78-82.

McCoy, R.E. 1983. Wall-free prokaryotes of plants and invertebrates. *In*: The Prokaryotes, vol. 2, p. 2238-2246, M.P. Starr, H. Stolp, H.G. Truper, A. Bolows and H.G. Schlegel (ed.), Springer-Verlag, New York Inc.

McCoy, R.E., Caudwell, A., Chang, C.J., Chen, T.A., Chiykowski, L.N., Cousin, M.T., Dale de Leeuw, G.T.N., Golino, D.A., Hackett, K.J., Kirkpatrick, B.C., Marwitz, R., Petzold, H., Shina, R.H., Sugiura, M., Whitcomb, R.F., Yang,

I.L., Zhu, B.M., and Seemüller, E. 1989. Plant diseases associated with mycoplasmalike organisms. *In*: The Mycoplasmas, vol. 5, p. 545-560, R.F. Whitcomb and J.G. Tully (de.), Academic Press, New York.

McGarrity, G.J., Steiner, T., and Vanaman, V. 1983. Detection of mycoplasmal infection of cell cultures by DNA fluorochrome staining. *In*: Methods in Mycoplasmology, vol. II Diagnostic Mycoplasmology, p. 183-190, J.G. Tully and S. Razin (ed.), Academic Press, New York.

Memane, S.A., and Joi, M.B. 1987. Identification of sources of resistance to little leaf of bringjal. J. Maharashtra Agric. Univ. 12, 70-71.

Mitsuhashi, J., and Marmorosch, K. 1964. Inoculation of plant tissue cultures with aster yellows virus. Virology 223, 277-279.

Mittempergher, L., Fagnani, A., Ferrini, F., and D'Agostino, G. 1990. Elm yellows, a disease to be taken into consideration when breeding elm for disease resistance. *In*: Proceedings of the 8th Congress of the Mediterranean Phytopathological Union. Agadir, Morocco.

Moellers, C., and Sarkar, S. 1989. Regeneration of healthy plants from *Catharanthus roseus* infected with mycoplasmalike organisms through callus culture. Plant Sci. 60, 83-90.

Mullis, K.B., Faloona, F., Scharf, S.J., Saiki, R.K., Horn, G.T., and Erlich, H.A. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51, 263-273.

Nakamura, H., Yoshikawa, N., Takahashi, T., Nahashi, N., Kubono, T., and Shoji, T. 1996. Evaluation of primer pairs for the reliable diagnosis of paulownia witches'-broom disease using a polymerase chain reaction. Plant Dis. 80, 302-305.

Nakane, P.K., and Pierce, G.G. 1966. Enzyme-labeled antibodies: preparation and application for the localization of antigens. J. Histochem. Cytochem. 14, 929-931.

Nakashima, K., Kato, S., Iwanami, S., and Murata, N. 1991. Cloning and detection of chromosomal and extrachromosomal DNA from mycoplasmalike organisms that cause yellow dwarf disease of rice. Appl. Environ. Microbiol. 57, 3570-3575.

Nakashima, K., Kato, S., Iwanami, S., and Murata, N. 1993. DNA probes reveal relatedness of rice yellow dwarf mycoplasmalike organisms (MLOs) and distinguish them from other MLOs. Appl. Environ. Microbiol. 59, 1206-1212.

Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H., and Tsuchizaki, T. 1993a. Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. Phytopathology 83, 786-791.

Namba, S., Oyaizu, H., Kato, S., Iwanami, S., and Tsuchizaki, T. 1993b. Phylogenetic diversity of phytopathogenic mycoplasmalike organisms. Int. J. Syst. Bacteriol. 43, 461-467.

Neimark, H.C., and Kirkpatrick, B.C. 1991. Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic mycoplasma-like organisms. Mol. Microbiol. 7, 21-28.

Petru, E., and Ulrychova, M. 1975. Persistence and spread of mycoplasma in axenic callus tissue cultures of tobacco (*Nicotiana glauca* Grah.) in the presence of kinetin and IAA in nutrient medium. Biol. Plant 17, 352-356.

Pollini, P.C., Bissani, R., Giumchedi, L., and Vindimian, E. 1996. First report of phytoplasma infection in olive trees (*Olea europea* L.). J. Phytopathol. 144, 109-111.

Prince, J.P., Davis, R.E., Wolf, T.K., Lee, I.-M., Mogen, B.D., Dally, E.D., Bertaccini, A., Credi, R., and Barba, M. 1993. Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. Phytopathology 83, 1130-1137.

Purcell, A.H. 1982. Insect vector relationships with prokaryotic plant pathogens. Ann. Rev. Phytopathol. 20, 397-417.

Raju, B.C., and Nyland, G. 1988. Chemotherapy of mycoplasma diseases of fruit trees. *In*: Tree Mycoplasmas and Mycoplasma Diseases, p. 207-216. C. Hiruki (ed.), University of Alberta Press, Edmonton, Alberta, Canada.

Razin, S. 1992. Mycoplasma taxonomy and ecology. *In*: Mycoplasmas: Molecular Biology and Pathogenesis, p. 3-22, J. Maniloff, R.N. McElhaney, L.R. Finch, and J. Baseman (ed.), American Society for Microbiology, Washington, D.C.

Rogers, M.J., Simmons, J., Walker, R.T., Weisburg, W.G., Woese, C.R., Tanner, R.S., Robinson, I.M., Stahl, D.A., Olsen, G., Leach, R.H., and Maniloff, J. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA 82, 1160-1164.

Russel, W.C., Newman, C., and Willianmson, D.H. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253, 461-462.

Saglio, P., Lafleche, D., L'Hospital, M., Dupont, G., and Bové, J.M. 1972. Isolation and growth of citrus mycoplasmas. *In*: Pathogenic Mycoplasmas: A CIBA Foundation Symposium, p. 187-203, Amersterdam, Elsevier-Excerpta Medica.

Saiki, R.D., Scharf, S., Saloona, F., Mullis, K., Horn, G., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350-1354.

Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1986. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature (Lond.) 324, 163-166.

Schaff, D.A., Lee, I.-M., and Davis, R.E. 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reaction. Biochem. Biophys. Res. Comm. 186, 1503-1509.

Schneider, B., Ahrens, U., Kirkpatrick, B.C., and Seemüller, E. 1993. Classification of plant-pathogenic mycoplasmalike organisms using restriction-site analysis of PCR-amplified 16S rDNA. J. Gen. Microbiol. 137, 519-527.

Schwartz, Y., Boudon-Padieu, E., Grange, J., Meignoz, R., and Caudwell, A. 1989. Monoclonal antibodies to the mycoplasmalike organism (MLO) responsible for grapevine flavescence doree. Res. Microbiol. 140, 311-324.

Sears, B.B., and Kirkpatrick, B.C. 1994. Unveiling the evolutionary relationships of plant-pathogenic mycoplasmalike organisms. Amer. Soc. Microbiol. News 60, 307-312.

Sears, B.B., and Klomparens, K.L. 1989. Leaf tip cultures of the evening primrose allow stable aseptic culture of mycoplasma-like organisms. Can. J. Plant Pathol. 11, 343-348.

Sears, B.B., Lim, P.-O., Holland, N., Kirkpatrick, B.C., and Klomparens, K.L. 1989. Isolation and characterization of DNA from a mycoplasmalike organism. Mol. Plant-Microbe Interact. 2, 175-180.

Seemüller, E. 1976. Fluoreszenzoptischer direktnachweis von mycoplasmaahnlichen organism in phloem pear-decline-und triebsuchtkranker bukme? Phytopathol. Z. 85, 368-372.

Seemüller, E., Schneider, B., Maurer, R., Ahrens, U., Daire, X., Kison, H., Lorenz, K.-H. Firrao, G., Avinent, L., Sears, B.B., and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int. J. Syst. Bacteriol. 44, 440-446.

Shaw, M.E., and Kirkpatrick, B.C. 1993. The beet leafhopper-transmitted virescence agent causes big bud disease in California. Plant Dis. 77, 290-295.

Shaw, M.E., Kirkpatrick, B.C., and Golino, D.A. 1990. Identification and characterization of plasmids present in the beet leafhopper transmitted virescence agnet. Phytopathology 80, 985 (Abstr.).

Shen, W.C., and Lin, C.P. 1993. Production of monoclonal antibodies against a mycoplasmalike organism associated with sweetpotato witches'-broom. Phytopathology, 83, 671-675.

Sinclair W.A., Griffiths, H.M., and Davis, R.E. 1996. Ash yellows and lilac witches'-broom: phytoplasmal diseases of concern in forestry and horticulture. Plant Dis. 80, 468-475.

Sinha, R.C. 1974. Purification of mycoplasma-like organisms from China aster plants infected with clover phyllody. Phytopathology 64, 1156-1158.

Sinha, R.C. 1988. Serological detection of mycoplasmalike organisms from plants affected with yellows diseases. *In*: Tree Mycoplasmas and Mycoplasma Diseases, p. 143-156, C. Hiruki (ed), University of Alberta Press, Edmonton, Alberta, Canada.

Sinha, R.C., and Benhamou, N. 1983. Detection of mycoplasmalike organisms antigens from aster yellows-diseased plants by two serological procedures. Phytopathology 73, 1199-1202.

Sinha, R.C., and Chiykowski, L.N. 1967. Initial and subsequent sites of aster yellows virus infection in a leafhopper vector. Virology 33, 702-708.

Sinha, R.C., and Chiykowski, L.N. 1984. Purification and serological detection of mycoplasmalike organism from plants affected by peach eastern X-disease. Can. J. Plant Pathol. 6, 200-205.

Sinha, R.C., and Chiykowski, L.N. 1990. Serological detection of MLOs in plants and vector leafhoppers using polyclonal antibodies. Zentralbl. Bakteriol. Suppl. 20, 276-279.

Steiner, T., McGarrity, G.J.,and Phillips, D.M. 1982. Cultivation and partial characterization of spiroplasmas in cell cultures. Intect. Immunity 35, 296-304.

Steiner, T., McGarrity, G.T., Bové, J.M., Phillips, D.M., and Garnier, M. 1983. Insect cell cultures in the study of attachment and pathogenicity of spiroplasmas and mycoplasmas. Ann. Microbil. (Inst. Pasteur) 135A: 47-53.

Strauss, S.H., Howe, G.T., and Goldfarb, B. 1991. Prospects for genetic engineering of insect resistance in forest trees. Forest Ecology and Management 43, 181-209.

Tang, K., Davey, M.R., Blackhall, N.W., Cocking, E.C., Power, J.B., Wang, M., Shi, Y., Edmonds, H., Powsell, K., Gatehouse, L., Hilder, V., Boulter, D., Gatehouse, A., and Gatehouse, J. 1994. Transformation of *Japonica* rice with an insect resistance gene driven by a phloem-specific promoter. Internat. Cong. Plant Mol. Biol. 14-24.

Tang, W., and Chen, T.A. 1992. Molecular cloning and detection of DNA for the mycoplasmalike organism associated with blueberry stunt disease. Phytopathology, 82, 248.

Thompson, L.S., Cutcliffe, J.A., Gourley, C.O., and Murray, R.A. 1973. Evaluation of several insecticides for control of strawberry green petal disease. Can. Plant Dis. Survey 53, 16-18.

Vega, F.E., Davis, R.E., Barbosa, P., Dally, E.L., Purcell, A.H., and Lee, I.-M. 1993. Detection of a plant pathogen in a nonvector insect species by the polymerase chain reaction. Phytopathology 83, 621-624.

Vibio, M., Bertaccini, A., Lee, I.-M., Davis, R.E., and Clark, M.F. 1994. Characterization of aster yellows and related European mycoplasmalike organisms maintained in periwinkle plants and shoot-tip culture. IOM Lett. 3, 297-298.

Wang, B., Merua M., Williams V., and Weiner B. 1995. Large-scale preparation of plasmid DNA by microwave lysis. Biotechniques, 18, 554-555.

Wang, K., Zhong, Q., Khadhair, A.H., and Hiruki, C. 1994. DNA amplification based on polymerase chain reaction for the sensitive detection of the mycoplasmalike organism associated with paulownia witches'-broom. Pro. Japan. Acad. 70B, 87-91.

Waters, H., and Hunt, P. 1980. The *in vivo* three-dimensional form of a plant mycoplasmalike organism by the analysis of serial ultrathin sections. J. Gen. Microbiol. 116, 111-131.

Weisburg, W.G., Tully, J.G., Rose, D.L., Petzel, J.P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., Maniloff, J., and Woese, C.R. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. 171, 6455-6467.

Whitcomb, R.F., and Black, L.M. 1982. Plant and arthropod mycoplasmas: a historical perspective. *In*: Plant and Insect Mycoplasma Techniques, p. 40-81, M.J. Daniels and P.G. Markham (ed.), Halster Press, New York.

Woese, C.R. 1987. Bacterial evolution. Microbiol. Rev. 51, 221-271.

Woese, C.R., Maniloff, J., and Zablen, L.B. 1980. Phylogenetic analysis of the mycoplasmas. Proc. Natl. Acad. Sci. USA 77, 494-498.

Wolanki, B., and Maramorosch, K. 1970. Negatively stained mycoplasmas :fact or artifact? Virology 42, 319-327.

Yoshikawa, N., Nakamura, H., Sahashi, N., Kubono, T., Katsube, K., Shoji, T., and Takahashi, T. 1994. Amplification and nucleotide sequences of ribosomal protein and 16S rRNA genes of mycoplasma-like organism associated with paulownia witches'-broom. Ann. Phytopathol. Soc. Japan 60, 569-575.

Zhong, Q., and Hiruki, C. 1994. Genetic differentiation of phytoplasma isolates determined by a DNA heteroduplex mobility assay. Proc. Japan. Acad. 70B, 127-131.

CHAPTER II

DNA AMPLIFICATION BASED ON POLYMERASE CHAIN REACTION FOR SENSITIVE DETECTION OF PHYTOPLASMA ASSOCIATED WITH PAULOWNIA WITCHES'-BROOM *

---

* A version of this chapter has been published. Wang, K., Zhong, Q., Khadhair, A.-H., and Hiruki, C. 1994. Proc. Japan Acad. 70B:87-91.

II.1 INTRODUCTION

Paulownia is an important and fast-growing tree species for production of high quality lumber in Asia and South America. It is distributed in more than twenty-three provinces in China alone and accounts for 1.3 billion trees. Paulownia witches'-broom (PaWB), the most serious disease of paulownia in China, Korea and Japan, is one of the first reported plant diseases associated with phytoplasmas and inflicts major economic losses (Doi *et al.*, 1967). While about 5-10% of seedlings are infected by PaWB phytoplasma within a year after transplanting in the field, the disease is known to increase to 50-80% 5 to 6 years later. The rate of premature death due to PaWB is about 20% for seedlings, whilst the growth rate of the diameter of trunks of PaWB-infected old trees is 23 to 29.5% less than that of healthy trees, the maximum reduction being 83% (Jin, 1982). Because no effective control measures were available in the past, PaWB disease spread nation-wide in China during the 1970s through 1980s. PaWB incidence in the early 1970s in northern China was about 10-20%; in the 1980s, more than 70% of 10-year-old paulownia trees were affected by PaWB phytoplasma (Jin, 1982). As cultivation of paulownia increased rapidly after the 1960s, PaWB became a prominent problem, threatening the development of paulownia silviculture (Tian and Raychaudhuri, 1996).

Sensitivity of detection is a key feature in preventing the development of PaWB disease. Molecular cloning of phytoplasma DNA and use of the cloned DNA fragments as hybridization probes have been utilized for the diagnosis of many phytoplasma diseases (Kirkpatrick *et al.*, 1987; Davis, M. J. *et al.*, 1988; Davis, R. E. *et al.*, 1988; Deng and Hiruki, 1991a). More recently, the development of polymerase chain reaction (PCR) has

provided the most sensitive and specific method for detecting phytoplasmas in infected plants (Deng and Hiruki,1990, 1991a, b; Schaff *et al.*, 1992; Davis *et al.*, 1992; Ahrens and Seemüller,1992; Lee *et al.*, 1993).

In this chapter, we report the application of PCR as a sensitive method for the detection of phytoplasmas maintained in paulownia tissue culture and in seedlings grown in the greenhouse, and establish an appropriate range of concentrations of phytoplasma DNA for PCR amplification. It was confirmed also that phytoplasma titers in infected tissue cultures were higher than those in infected plants maintained under greenhouse conditions.

II.2 MATERIALS AND METHODS

II.2.1 Sources of Healthy and Diseased Paulownia Plants.

Phytoplasma-infected tissue culture plantlets were established as follows; fresh, tender twigs with typical symptoms of PaWB were washed in tap water. The petiole tissue 1cm long was left attached to protect the axillary bud from damage. The stems were cut into segments 5-8 cm long, surface sterilized by soaking in 75% ethanol for 30 seconds, washed in sterile distilled water, then soaked in 1% hypochlorite solution for 5-7 minutes, washed in sterile distilled water thoroughly, air-dried or blotted with sterile filter paper. Stems about 0.5 cm long with a pair of axillary bud were transferred to MS medium (Murashige and Skoog, 1962) without hormone. The plant tissues were kept in an incubator at 25-28°C for a 16 hr photoperiod with light intensity maintained at 1200-1500 Lux. Healthy paulownia was grown from seed. Diseased plants were established

from phytoplasma-infected paulownia roots and were maintained in the greenhouse.

II.2.2 Extraction of Nucleic Acids from Plants

DNA samples from healthy plants and plants infected with PaWB phytoplasma were prepared by a modified procedure of Ausubel *et al.* (1987). Each sample, about 1.0g of the whole tissue culture or leaf midribs cut from the plants grown in greenhouse, was pulverized in liquid nitrogen in a mortar. The powder was triturated in 10ml of DNA extraction buffer [100mM Tris-HCl (pH 8.0),100mM EDTA, 250mM NaCl, 100mg of proteinase K per ml]. To this sample, 1ml of 10% sodium dodecyl sulfate (SDS) and 0.5ml 20% sarkosyl were added. The sample was incubated at 55 °C for 1 hr and then centrifuged at 3,000*g* for 10 min. to separate debris. Crude DNA was precipitated from the supernatant by adding 2 vol. of 95% ethanol and placed at -20 °C overnight to precipitate DNA. After centrifugation at 16,000*g* for 15 min, the pellet was resuspended in 1 ml of TE buffer (10mM Tris-HCl, 1mM EDTA [pH8.0]) containing 100 mg of proteinase K per ml and 0.5% SDS and incubated at 37 °C for 1 hr, followed by the addition of 175 ml of 5M NaCl and 140 ml of 10% cetyltrimethyl-ammonium bromide (CTAB) in 0.7M NaCl. The mixture was incubated at 65 °C for 10 min, then extracted once with an equal volume of tris-saturated phenol and an equal volume of chloroform-isoamyl alcohol (24:1). The sample was then washed at least twice with 2 volumes of chloroform-isoamyl alcohol (24:1). Two volumes of 95% ethanol and 1/10 volume of 3M NaOAc was added to the aqueous phase and placed at -70 °C for at least 1 hr. The DNA was pelleted by centrifugation at 16,000*g* for 15 min. The pellet was then washed with

70% ethanol, dried under vacuum, and resuspended in 1ml TE buffer.

II.2.3 PCR Primers

Two oligonucleotide primers (Lee *et al.*, 1993) were chosen for this study because they can amplify a 16S rDNA fragment from DNA of phytoplasma infected tissues but not from any DNA sample extracted from healthy tissues. The oligonucleotide sequences of the two primers and base locations are R16F2, 5'-ACGACTGCTGCTAAGACTGG-3' (base 152-168); R16R2, 5'-TGACGGGCGGTGTGTACAAACCCCG-3' (base 1373-1397).

II.2.4 PCR Amplification

Total DNA fragments were amplified from 10µl of 2-fold serially diluted DNA samples extracted from phytoplasma-infected paulownia tissue cultures, phytoplasma-infected paulownia plants, and healthy plants grown in the greenhouse. The amplification was performed in a 100µl PCR reaction mixture containing 200µM each of dATP, dCTP, dGTP, and dTTP, 1µM of each upstream and downstream primer, 10µl of 10× PCR reaction buffer and 2.5mM $MgCl_2$ (Promega Corp.), 2.5U *Taq* DNA polymerase (Promega Corp.), and 30µl mineral oil. The PCR was carried out for 45 cycles in a Biooven (BioTherm, Virginia) under the following conditions: 1st cycle, denaturation 5 min at 94 °C; annealing 2 min at 50 °C; extension 3 min at 72 °C; 43 cycles, denaturation 1 min at 94 °C; the final cycle, extension for 7 min at 72 °C. Denaturation and annealing conditions were the same as for the first cycle.

II.2.5 Agarose Gel Electrophoresis of PCR Products

PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

II.3 RESULTS AND DISCUSSION

When the total nucleic acids used for PCR were diluted from 2-fold to 2^{20}-fold, the phytoplasma-specific band representing a DNA fragment size of 1.2kb was observed in ethidium bromide-stained agarose gel which corresponded to the reaction mixtures containing as little as 16pg of total nucleic acid from PaWB phytoplasma-infected tissue. No band was observed when a sample containing only DNA from healthy plants was examined (Fig.II.1). However, when the total nucleic acids from phytoplasma-infected tissue were diluted from 2-fold to 2^{6}-fold, the amplification by PCR was not consistent, because phytoplasma-specific amplification by PCR was interfered by non-target host plant DNA and presumptive reaction inhibitors in the PCR mixture (Schaff *et al.*, 1992). The amplification by PCR was consistent when the total DNA was diluted from 2^{7}-fold to 2^{16}-fold, especially from 2^{8}-fold to 2^{12}-fold. In contrast, no consistent amplification by PCR resulted when total DNA was diluted beyond 2^{16}-fold. In terms of the concentration of total DNA, the most appropriate range for amplification by PCR was 240pg/ml to 6.5ng/ml.

The original concentration of the total nucleic acids directly extracted from tissue

culture and intact plants was 1.7mg/ml and 800ng/ml, respectively. The 16S rDNA fragment was amplified until the total nucleic acids from tissue culture was diluted 10^6-fold, while that from plants was diluted 10^5-fold (Fig.II.2). The conclusion was that the PaWB phytoplasma titer in paulownia tissue culture was significantly higher than that in plants grown under greenhouse conditions. The presence of phytoplasmas at a high level in *Oenothera* leaf tip culture has been reported previously (Sears and Klomparens, 1989).

PCR has been successfully employed for specific phytoplasma detection (Deng and Hiruki, 1990, 1991a and b; Schaff *et al.*, 1992; Davis *et al.*, 1992; Ahrens and Seemüller, 1992; Lee *et al.*, 1993). In many studies, phytoplasma DNA was extracted from test plants other than their original host plants, e.g. the phytoplamsa DNA was extracted from the infected periwinkle after dodder transmission from the original host plant (Deng and Hiruki, 1990, 1991a and b; Davis et al., 1992; Ahrens and Seemüller, 1992). This was more time-consuming than the direct detection of phytoplasmas from original host plants. In this report, the phytoplasma DNA used for PCR for detection of PaWB phytoplasma was directly extracted from paulownia plants showing witches'-broom symptoms. This makes it possible to perform simple detection of paulownia witches'-broom using the original disease samples.

When used for detection of PaWB phytoplasma DNA in low concentration in paulownia trees, the PCR amplification can serve as a diagnostic method with high sensitivity. It can detect as little as 16pg of total nucleic acid in the PCR mixture from phytoplasma-infected paulownia plants. The phytoplasmas exist only in the phloem tissue of infected plants, and the phytoplasma titers per unit fresh weight of tissue are generally low (Kollar *er al.*, 1990). Phytoplasma DNA may constitute less than 0.1% of total DNA

in extracts from woody hosts exhibiting disease symptoms (Kirkpatrick, 1989). The concentration of phytoplasmas in infected, but asymptomatic, plants may be much less. Therefore, the high sensitivity of PaWB phytoplasma detection by PCR will prove very important for practical use in field diagnosis of disease specimens.

Fig.II.1. Polymerase chain reaction (PCR) analysis of DNA extracted from healthy paulownia plants, tissue culture of witches'-broom affected paulownia and paulownia plants showing witches'-broom symptoms. The 1.5% agarose gel was stained with ethidium bromide and the bands were visualized with UV light. Lane 1, 123-bp DNA ladder. Lane 2, nucleic acids from healthy paulownia. Lane 3, undiluted total nucleic acids from PaWB phytoplasma-infected paulownia tissue culture. Lanes 4 to 23, total nucleic acids from PaWB phytoplasma-infected paulownia tissue culture were diluted sequentially from 2-fold to 2^{20}-fold. The nucleic acids were used as template for each PCR amplification. The concentration of total nucleic acids in the original extracts was 1.7mg/ml.

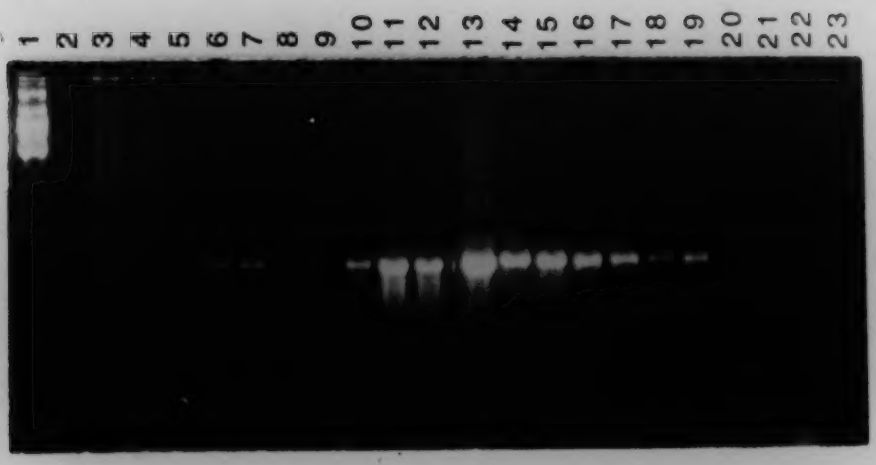

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

Fig.II.2. Comparison of phytoplasma content in phytoplasma-infected tissue culture maintained at 25-28°C and in phytoplasma-infected paulownia plants grown at 23-25 °C under greenhouse conditions. Lane 1, 123-bp DNA ladder. Lane 2, total nucleic acids from healthy paulownia. Lanes 3 to 12, total nucleic acids from PaWB phytoplasma-infected paulownia tissue culture, and lanes 13 to 22, total nucleic acids from phytoplasma-infected paulownia plants grown under greenhouse conditions at 23-25°C. The total nucleic acids were diluted sequentially from 10-fold to 10^{10}-fold and were used as template for each PCR amplification.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

II.4 REFERENCES

Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82, 828-832.

Ausubel, F. M. 1987. Current protocols in molecular biology, p. 1.6.2-1.6.4, Wiley Interscience, New York.

Davis, M. J., Tsai, J.H., Cox, R.L., Mcdaniel, L.L., and Harrison, N.A. 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize brushy stunt disease. Mol. Plant-Microbe Interact. 1, 295-302.

Davis, R. E., Lee, I.-M., Douglas, S.M., Dally, E.L., and DeWitt, N.D. 1988. Cloned nucleic acid hybridization probes in detection and classification of mycoplasmalike organisms (MLOs). Acta Hort. 234, 115-122.

Davis, R. E., Prince, J.P., Hammond, R.W., Dally, E.L., and Lee, I.-M. 1992. Polymerase chain reaction detection of Italian periwinkle virescence mycoplasmalike organism (MLO) and evidence for relatedness with aster yellows MLOs. Petria 2, 184-193.

Deng, S., and Hiruki, C. 1990. Enhanced detection of a plant pathogenic mycoplasma-like organism by polymerase chain reaction. Proc. Japan Acad. 66, 140-144.

Deng, S., and Hiruki, C. 1991a. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81, 1475-1479.

Deng, S., and Hiruki, C. 1991b. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Methods 14, 53-61.

Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or P.L.T. group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches'-broom, aster yellows or paulownia witches'-broom. Ann. Phytopathol. Soc. Janpan 33, 259-266.

Jin, K. X. 1982. Study on paulownia witches'-broom. *In*: Collected Letters on Paulownia, p.116-126, Chinese Forest Soc. (Ed.), Chinese Forestry Publishing Co., Beijing.

Kirkpatrick, B.C. 1989. Strategies for characterizing plant pathogenic mycoplasma-like organisms and their effects on plants. *In:* Plant-Microbe Interactions, Molecular and Genetic Perspectives, vol. 3, p. 241-293, T. Kosuge and E.W. Nester (ed.), Mcgraw-Hill, New York.

Kirkpatrick, B. C., Stenger, D.C., Morris, T.J., and Purcell, A.H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasmalike organisms. Science, 238, 197-200.

Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bové, J.M. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. Phytopathology 80, 233-237.

Lee, I.-M., Hammond, R.W., Davis, R.E., and Gundersen, D.E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834-842.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473-497.

Tian, G., and Raychaudhuri, S.P. 1996. Paulownia witches'-broom disease in China: present status. *In*: Forest Trees and Palms, Diseases and Control, p. 227-251, S.P. Raychaudhuri, and K. Maramorosch (ed.), Oxford & IBH Publ. Co. PVT. LTD., New Delhi.

Schaff, D. A., Lee, I.-M., and Davis, R.E. 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reaction. Biochem. Biophys. Res. Commun. 186, 1503-1509.

Sears, B. B., and Klomparens K. L. 1989. Leaf tip cultures of the evening primrose allow stable, aseptic culture of mycoplasmalike organism. Can. J. Plant Pathol., 11, 343-348.

CHAPTER III

PRESERVATION OF PHYTOPLASMA DNA IN MICROWAVE-DRIED TISSUES *

---

* A version of this chapter has been accepted for publication and is in press.

Wang, K., and Hiruki, C. 1997. J. Microbiol. Methods

III.1 INTRODUCTION

Phytoplasmas (formerly called mycoplasma-like organisms) are wall-less prokaryotes and are believed to be the causal agents of over three hundred plant diseases of economically important crops throughout the world (McCoy *et al.*, 1989). Although the phytoplasmas cannot be cultured *in vitro*, significant progress has been made on their classification and characterization by molecular approaches, such as nucleic acid hybridization (Al-Hakim and Hull, 1988; Kirkpatrick and Davis, 1988), biotinylated DNA probes, and polymerase chain reaction (Deng and Hiruki, 1990, 1991a; Lee *et al.*, 1993). Several methods have been reported for preservation of phytoplasmas including the maintenance in plant tissue cultures (Cousin, 1990; Sears and Klomparens, 1989), in living host plants (Markham, 1982), in leafhopper vectors below -70°C (Chiykowski, 1988), and freeze drying. All these methods are appropriate and useful, but they are time-consuming, expensive, and sometimes are not suitable for exchanging phytoplasma-infected tissues with other international research centers. In addition, monoclonal antibodies produced against periwinkle-maintained paulownia witches'-broom did not cross-react with phytoplasmas on infected paulownia (Chen, Y.X. personal communication). On the basis of Southern blot and 16S rDNA analyses, some other periwinkle-maintained phytoplasma isolates, widely used for maintenance of phytoplasmas *in vivo*, were found genetically different from those in naturally infected plants (Ahrens *et al.*, 1993; Schneider *et al.*, 1993). Therefore, a simple, economic, and practical method needs to be developed for maintenance of phytoplasmas for future research.

In a recent study, the molecular stability of clover proliferation phytoplasma DNA was revealed after a brief heat treatment of infected periwinkle plant tissues in a cooking microwave oven (Khadhair et al., 1995). However, the nucleic acids used in the previous study were extracted from infected periwinkle plants, and no molecular evidence has been available to show that the intactness of phytoplasma DNA was not affected by brief microwave treatment. The purpose of this study is to find out if this method can be extended to the preservation of phytoplasma DNA in woody tissue such as paulownia witches'-broom without affecting the genomic structure of the phytoplasma. Paulownia witches'-broom is one of the most important diseases of paulownia trees in China, Japan and Korea, yet there is no useful method for preservation of infected tissues of this species except in the living host or by tissue culture. In this chapter we report a suitable procedure for preparation of dehydrated paulownia tissues by microwave treatment for subsequent genetic studies of the phytoplasma DNA.

III.2 MATERIALS AND METHODS

III.2.1 Phytoplasma-infected Plant Tissues

Diseased paulownia plants were grown in the greenhouse and in tissue culture from field-collected paulownia shoots showing typical witches'-broom symptoms. Healthy tissues were started from paulownia seed. The tissue culture conditions were used as described previously (Chapter II).

III.2.2 Microwave Treatment and Air Dry

Diseased paulownia tissue cultures, midribs and petioles, 0.5 g each, were subjected to the heat-treatment for 1, 2, 4, 6, 8, 10, 12. 14, 16, 20 min respectively in a cooking microwave oven (2450 MHz, 600 W, Camco, Model: JM1026, Korea) at power 10 or were air-dried and maintained at room temperature (25 °C) for one month. The dehydrated materials were kept at room temperature for four weeks before extracting the nucleic acids. Fresh tissues, 0.5 g each, from both healthy and diseased plantlets were used for extraction of standard DNA.

III.2.3 DNA Extraction

Total genomic DNA from both dehydrated and fresh tissues were extracted by a modified procedure previously described (Kollar *et al.*, 1990). The dehydrated samples were pulverized in liquid nitrogen in mortars and pestles. Each ground tissue sample was homogenized in 3 ml of DNA extraction buffer [2.5M NaCl, 0.5% (W/V) PVP-10 (polyvinylpyrrolidone-10), 1% (W/V) cetavlon (hexadecyltrimethyl-ammonium bromide), 0.5M Tris-HCl (pH 8.0), 0.2% 2-mercaptoethanol]. The samples were then incubated at 65°C for 2 hours and centrifuged at 10,000*g* for 10 min. The supernatant was mixed with 2 volumes of chloroform-isoamyl alcohol (24:1). After centrifugation at 12,000*g* for 10 min, the DNA was precipitated by addition of 0.7 volume of isopropanol and 0.1 volume of 3M NaOAc to the aqueous phase at -70 °C overnight. Subsequently, the final pellets were washed with 70% ethanol, dried under vacuum and suspended in 100 µl TE buffer (pH 8.0). The DNA preparations were then treated with DNase-free RNase and proteinase K separately and stored at 4°C for molecular analyses.

III.2.4 DNA Amplification

A pair of universal primers R16F2 and R16R2 was used for amplification of a 1.2 kb DNA fragment (Lee *et al.*, 1993). The DNA samples were appropriately diluted before using for PCR amplification. The reaction mixture (100 µl) contained 200 µM each of dNTP's, 1 µM of each upstream and downstream primer, 1X PCR reaction buffer, 2.5mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promega). The reaction mixture was covered with 30 µl mineral oil. The amplification was carried out using 35 thermal cycles in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The following thermal conditions were applied: denaturation at 94°C for 1 min (5 min for the first cycle), annealing at 50°C for 2 min, extension at 72°C for 3 min. The final cycle was extended for 7 min at 72°C. About 10 µl of each PCR product was analyzed in a 1% (w/v) agarose gel electrophoresis followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

III.2.5 Restriction Fragment Length Polymorphism (RFLP)

A 5 µl aliquot of PCR products representing each treatment was digested with the restriction endonucleases *Alu*I and *Hha*I according to the instructions of the manufacturer (GIBCO BRL, Burlington). The restriction products were analyzed by electrophoresis in a 5% (w/v) polyacrylamide gel in Tris-Borate (TBE) buffer followed by staining in ethidium bromide. The fragments were observed and photographed under a UV transilluminator.

III.2.6 DNA Sequencing

The 16S rDNA fragment (about 1.5 kb) amplified from PaWB phytoplasma DNA derived from 20 min-microwave-treated tissues or fresh tissues using a primer pair P1/P6 (Deng and Hiruki, 1991b)was purified using a PCR clean up kit (Boehringer Mannheim Biochemica, Germany) and used as a template of DNA sequencing. Enzymatic DNA sequencing of a double-stranded template was carried out with *Taq* DNA polymerase (GIBCO BRL, Burlington) as described (Innis *et al.* 1988).

III.3 RESULTS AND DISCUSSION

The color of tissue cultures remained green after microwave treatment, while midrib and petiole tissues changed from green to brown after air-drying. This discoloration of air-dried samples might be due to some chemical reactions induced by enzymes such as peroxidase and polyphenol oxidase etc. which are abundant in paulownia trees (Tian and Raychaudhuri, 1996). The genomic DNA bands were not detectable in a 1% (w/v) agarose gel when samples were extracted from air-dried tissue cultures, midribs, and petioles. The amount of total DNA from microwave-treated midribs and petioles from plants grown in the greenhouse was relatively low, whereas that from microwave-treated tissue cultures was high (Fig.III.1). This result not only confirmed the results of the previous study in which the concentration of phytoplasma in tissue cultures was much higher than that in intact host plants in the field (Chapter II), but also suggested that the genomic DNAs were damaged during air-drying of samples, or alternatively bound to host cellular components, or both.

Since the phytoplasma-infected cells are subjected to dehydration by microwave, it is possible that nucleic acids are bound to some cellular components. The amount of DNA extracted from these tissues tended to be very low when the standard DNA extraction protocol was used. Further improvements in this extraction procedure are required to ensure better DNA release from the cellular complex.

Prominent bands of 1.2 kb were observed on a 1% (w/v) agarose gel after amplification of DNA samples from all the samples consisting of microwave-treated tissue cultures, midribs and petioles up to 14 min, and fresh phytoplasma-infected paulownia tissue cultures (Fig.III.2). No DNA fragment was amplified from air-dried samples, microwave-treated midribs and petioles after 16 min, and fresh healthy samples. The amplification of the DNA with specific bands matching the size of the universal primers indicated that the microwave-treatment had no adverse effect on the molecular structure of the DNA in the dehydrated tissues. If there were such an effect, it would have resulted in the formation of several non-specific bands. The reason for no amplification from microwave-dried midribs and petioles after 16 min was probably due to the low DNA content in the samples that could not be extracted from over-heated materials. The lowest DNA concentration required to achieve PCR-positive results was approximately 16pg/µl when paulownia tissue cultures were used as a source material under similar experimental conditions (Chapter II). The RFLP analyses of all amplified DNA fragment with *Alu*I and *Hha*I enzymes showed identical or very similar RFLP patterns for both dehydrated by microwave and fresh infected samples (Fig.III.3). The 16S rDNA sequences also revealed that there was no difference between microwave-treated and untreated phytoplasma DNA (Fig.III.4). All the above analyses provide conclusive

evidence for the stability of the molecular structure of the phytoplasma DNA under the conditions of this study.

The plant tissues subjected to the microwave heat treatment showed gradual decreases in dry weight as the dehydration time increased, after a sharp decline following 2 to 4 min heating (Fig.III.5). Therefore, the optimum time for dehydration of the tissues is from 6 to 10 min for phytoplasma DNA preservation in paulownia tissues. Biological viability of phytoplasma under the microwave conditions of this study is extremely short. A phytoplasma-infected *Catharanthus roseus* shoot, about 3 cm long, lost its viability even after a 10 second microwave-heat-treatment. Therefore, this method will not only offer a suitable means for preservation of phytoplasma DNA useful for molecular diagnosis but also for safe international exchange of experimental materials without violating quarantine regulations.

Fig.III.1. Agarose gel (1% w/v) electrophoresis of total DNA samples extracted from infected fresh paulownia tissue cultures, microwave-treated tissue cultures and midribs, air-dried tissue cultures and midribs. Lane 1, fresh tisue culture; lanes 2 to 11, microwave-treated tissue cultures for 1, 2, 4, 6, 8, 10, 12, 14, 16 and 20 min, lanes 12 to 21; microwave-treated midribs for 1, 2, 4, 6, 8, 10, 12, 14, 16 and 20 min; lanes 22, 23, air-dried tissue cultures and midribs respectively.

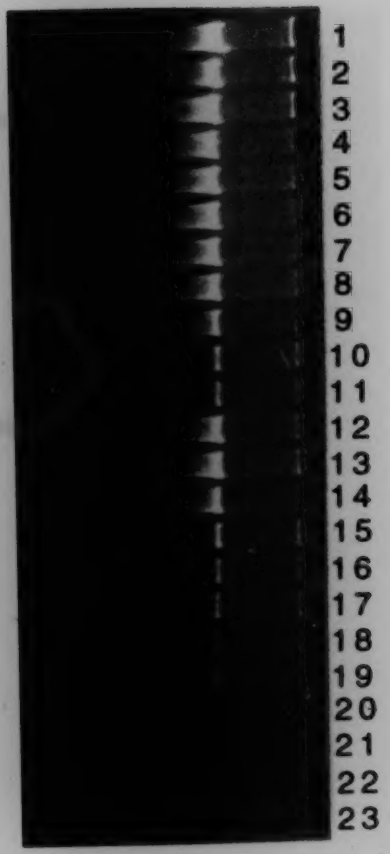

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

Fig.III.2. PCR amplification of 16S rDNA fragment (1.2 kb) from genomic DNAs extracted from microwave-treated, fresh healthy and phytoplasma-infected paulownia tissues using a primer pair R16R2/F2. A 10 µl aliquot of each PCR product was analyzed by electrophoresis in 1% (w/v) agarose gel. Lanes 1, 24, 100 bp DNA ladder; lane 2, fresh healthy tissues; lane 3, fresh diseased tissues; lanes 4-13, dehydrated tissue cultures for 1, 2, 4, 6, 8, 10, 12, 14, 16 and 20 minutes; lanes 14-22, dehydrated midribs for 2, 4, 6, 8, 10, 12, 14, 16 and 20 min, respectively; lane 23, air-dried tissue cultures.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

Fig.III.3. Restriction fragment length polymorphism analysis. A 5 µl aliquot of PCR products was digested with *Alu*I (upper) and *Hha*I enzymes (lower) and separated in 5% (w/v) polyacrylamide gel electrophoresis. Lanes 1, 100 bp DNA ladder; Lane 2, fresh tissue; Lanes 3 to 12, represent dehydrated tissue cultures for 1, 2, 4, 6, 8, 10, 12, 14, 16, 20 min, respectively.

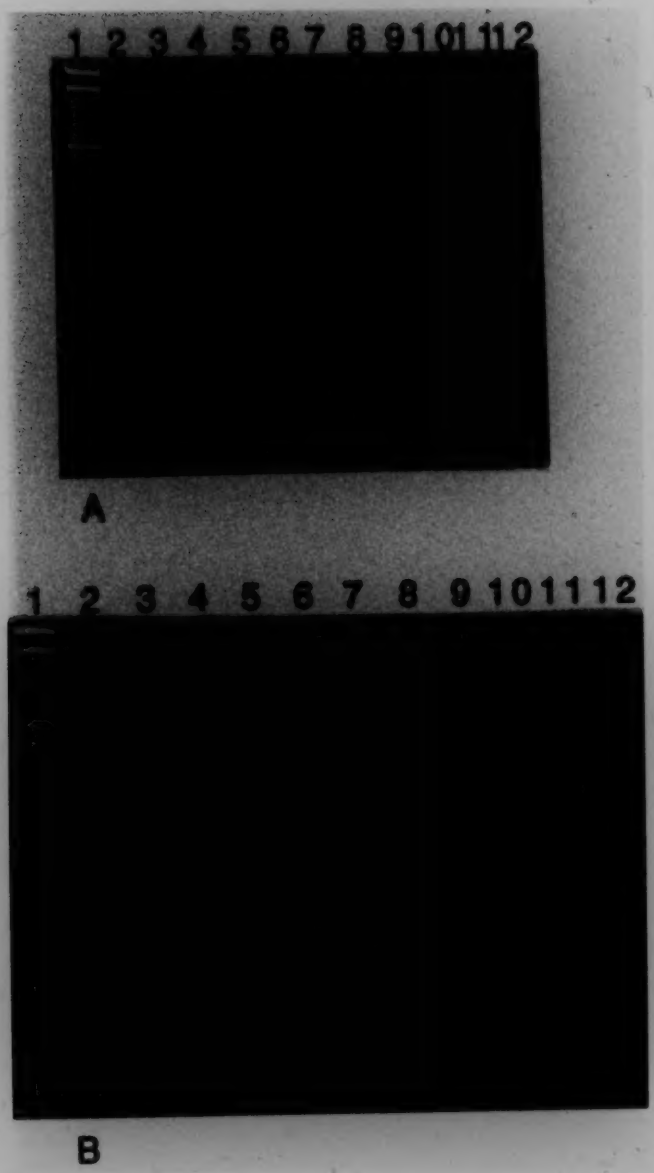
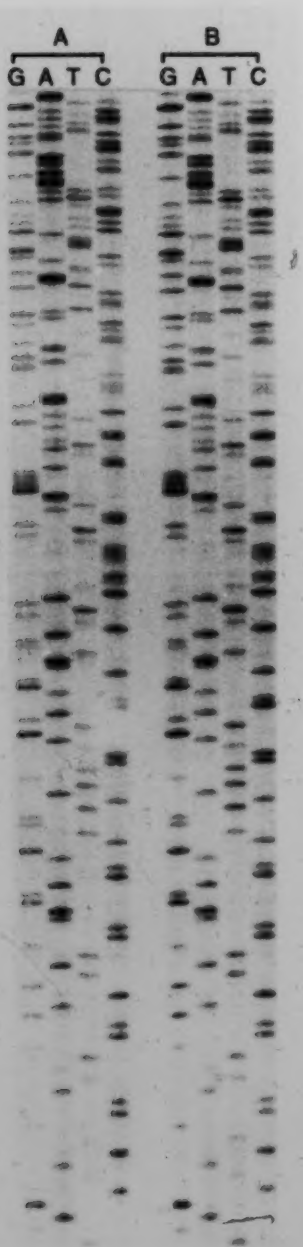

1 2 3 4 5 6 7 8 9 10 11 12
A
1 2 3 4 5 6 7 8 9 10 11 12
B

Fig.III.4. Sequencing results produced from amplified DNA from microwave-treated tissue cultures (20 min) and from untreated paulownia tissues showing typical witches'-broom symptoms.


A
G A T C
B
G A T C

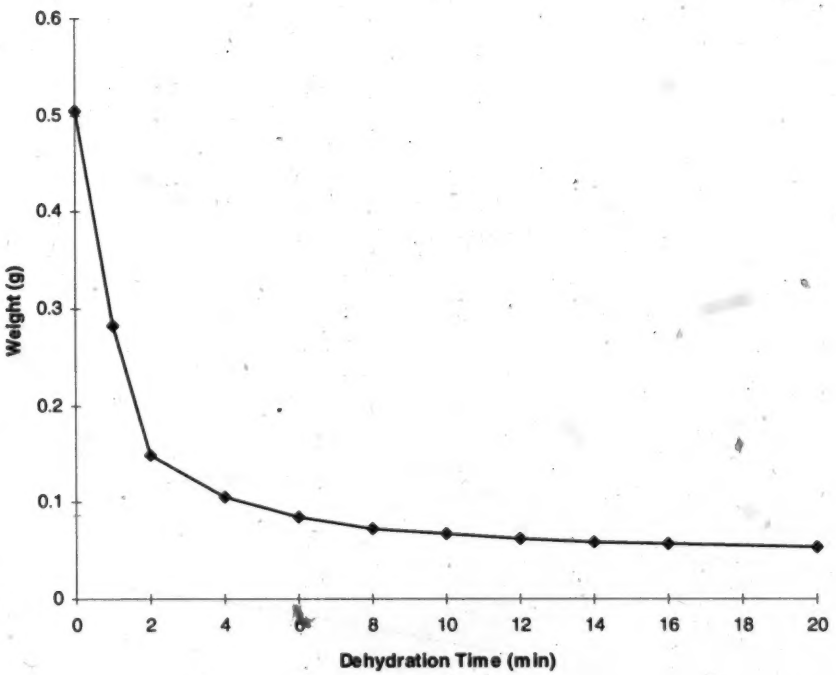


Fig.III.5. Relationship between dehydration time and the dry weight of plant tissues after microwave heat-treatment

I.4 REFERENCES

Ahrens, U., Lorenz, K.H., and Seemüller, E. 1993. Genetic diversity among mycoplasma-like organisms associated with stone fruit diseases. Mol. Plant-Microbe Interact. 6, 686-691.

Al-Hakim, A., and Hull, R. 1988. Chemically synthesized nonradioactive biotinylated long-chain nucleic acid hybridization probes. Biochem. J. 251, 935-938.

Chiykowski, L.N. 1988. Maintenance of yellows-type mycoplasmalike organisms. *In*: Tree Mycoplasmas and Mycoplasma Diseases, p. 123-134, C. Hiruki, (ed.), University of Alberta Press, Edmonton.

Cousin, M.-T., Roux, J., Millet, N., and Michel, M.-F. 1990. Maintenance of MLOs (mycoplasma-like organisms) on *Populus alba* micropropagation. J. Phytopathol. 130, 17-23.

Deng, S., and Hiruki, C. 1990. Enhanced detection of plant pathogenic mycoplasma-like organism by polymerase chain reaction. Proc. Japan Acad. 66B, 134-138.

Deng, S., and Hiruki, C. 1991a. Genetic relatedness between two nonculturable mycoplasma-like organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81, 1475-1479.

Deng, S., and Hiruki, C. 1991b. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. J. Microbio. Methods 14, 53-61.

Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A.D. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Froc. Natl. Acad. Sci. USA 85, 9436-9440.

Khadhair, A-H., Momani, F., and Hiruki, C. 1995. Molecular stability of clover proliferation phytoplasma DNA in periwinkle plant tissues after thermal treatment under microwave conditions. Proc. Japan Acad. 71B, 265-268.

Kirkpatrick, B.C., and Davis, M.J. 1988. Cloning and detection of DNA from the Western X-disease and maize bushy stunt mycoplasma-like organisms. Proc. 5th Intl. Con Plant Pathol. p.91, Kyoto, Japan (Abstract).

Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bové, J.M. 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Phytopathology 80, 233-237.

Lee, I-M., Hammond, R.W., Davis, R.E., and Gundersen D.E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopatholgoy 83, 834-842.

Markham, P.G. 1982. The 'yellows' plant diseases: plant hosts and their interaction with the pathogens. *In:* Plant and Insect Mycoplasma Techniques, p. 82-100, M.J. Daniels and P.G. Markham, (ed.), Croom Helm Ltd., London.

McCoy, R.E., Caudwell, A., Chang, C.J., Chen, T.A., Chiykowski, L.N., Cousin, M. T., Dale, J.L., De Leeuw, G.T.N., Golino, D.A., Hackett, K.J., Kirkpatrick, B.C., Marwitz, R., Petaold, H., Sinha, R.C., Sugiura, M., Whitcomb, R.F., Yang, I.L., Zhu, B.M., and Seemüller, E. 1989. Plant diseases associated with mycoplasma-like organisms. *In*: The Mycoplasmas, Vol. 5, p.540-545, V.R.F. Whitcomb and J.G. Tully, Eds. Academic Press, San Diego.

Schneider, B., Ahrens, U., Kirkpatrick, B.C., and Seemüller, E. 1993. Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. J. General Microbiol. 139, 519-527.

Sears, B.B., and Klomparens, K.L. 1989. Leaf tip cultures of the evening primrose allow stable, aseptic culture of mycoplasmalike organism. Can. J. Plant Pathol. 11, 343-348.

Tian, G., and Raychaudhuri, S.P. 1996. Paulownia witches'-broom disease in China: present status. *In*: Forest Trees and Palms, Diseases and Control, p. 227-251, S.P. Raychaudhuri and K. Maramorosch (ed.), Oxford & IBH Publ. Co. PVT. LTD., New Delhi.

CHAPTER IV

GENETIC RELATEDNESS OF PHYTOPLASMAS OCCURRING IN PAULOWNIA AND JUJUBE IN ASIA *

IV.1 INTRODUCTION

Phytoplasmas induce diseases in many forest and fruit tree species (Hiruki, 1988a). Eight paulownia species (*Paulownia elongata, P. catalpifolia, P. coreana, P. tomentosa, P. taiwaniana, P. kawakamii, P. fortunei, and P. fargesii*) are believed to be affected by phytoplasmas (Tian and Raychaudhuri, 1996). Paulownia witches'-broom (PaWB), one of the first reported plant diseases caused by phytoplasmas (Doi *et al.*, 1967), is historically the most serious disease on paulownia trees and is prevalent in China (Tsai *et al.*, 1988), Japan (Doi and Asuyama 1981) and Korea (La, 1987). PaWB is generally characterized by the proliferation of axillary or lateral buds on new branches in the current season, followed by the production of secondary and tertiary branches, which produce chlorotic and spindly leaves (Tsai *et al.*, 1988). It is reportedly transmitted by insects, *Cyrtopeltis tenuis* (La, 1968; Jin, 1983), *Halyomorpha mista* Uhler (Shiozawa *et al.*, 1986) and *H. holys* (Shao *et al.*, 1982), and dodder (Jin, 1982). Although the symptoms and insect vectors of PaWB are similar in China, Japan, and Korea, the molecular evidence of their genetic relatedness has not been presented. Recently, on the basis of 16S ribosomal RNA (16Sr) and ribosomal protein gene analyses by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), a worldwide collection of phytoplasmas were classified into 11 groups in which PaWB phytoplasma in Taiwan was classified into subgroup 16Sr I-D (Lee *et al.* 1993; Gundersen *et al.* 1994). In a separate study, using the nucleotide sequence of 16S rRNA genes, Japanese phytoplasmas were divided into three groups and PaWB phytoplasma was found to be

very similar to *Oenothera hookeri* phytoplasma in group I together with onion yellows, tomato yellows and mulberry dwarf phytoplasmas (Namba *et al.*, 1993).

Another phytoplasma disease of fruit trees is jujube witches'-broom (JWB) (Chi, 1951). JWB is also widespread and is devastating plantations in China (Tsai *et al.*, 1988), Japan (Doi and Asuyama, 1981), and Korea (La, 1987;). One Chinese JWB phytoplasma isolate was reported to be closely related to elm yellows phytoplasma which both belong to group V (Kirpatrick *et al.* 1994). Until now, there has been no information on the genetic relatedness of the JWB phytoplasma isolates in different geographic areas and their relationship to PaWB phytoplasma although the symptoms of JWB occurring in these three countries are very similar.

Paulownia is a fast-growing tree and one of the best timber species in the world (Jin, 1982), while jujube is an important economic fruit tree. Their plantations are often close to one another. Therefore, the lack of information on genetic relatedness between PaWB and JWB phytoplasmas is not only a major obstacle in diagnosis, control and understanding their epidemiology but also in developing effective measures for their quarantine regulations. This chapter deals with PCR-based amplification of 16S rRNA genes of PaWB and JWB phytoplasma isolates from China, Japan, and Korea using two pairs of universal primers. The combined analyses of restriction fragment length polymorphism (RFLP) and the nucleotide sequence data were used to study the homology of PaWB and JWB phytoplasmas from China, Japan, and Korea, and polygenetic diversity of phytoplasmas.

IV.2 MATERIALS AND METHODS

IV.2.1 Sources of Healthy and Diseased Plants

Samples from PaWB-diseased paulownia trees were collected in Henan, China; Ibaraki and Iwate, Japan; and Chonbuk, Korea. Samples from JWB-infected jujube were collected in Beijing, China; Uji, Japan; and Chonbuk, Korea. All samples showed typical witches'-broom symptoms such as proliferation, yellows and little leaves. Healthy paulownia and jujube were sampled in China. Clover proliferation (CP), alfalfa witches'-broom (AWB) were collected in central Alberta, Canada. Phytoplasmas associated with aster yellows (AY27) and potato witches'-broom (PWB), originally collected in the central Alberta, were maintained in periwinkle under greenhouse conditions (Hiruki, 1988b). Elm yellows (EY) maintained in periwinkle was collected from New York.

IV.2.2 Extraction of Nucleic Acids from Plants

DNA samples were prepared from healthy and infected plants as previously described (Chapter II). The nucleic acids were treated with DNase-free RNase and followed by treatment with proteinase K. The resulting DNA fractions were precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA) to a final concentration of 100 μg/ml.

IV.2.3 Primers and PCR Conditions

Two sets of universal primer pairs, P_1, 5'-AAGAGTTTGATCCTGGCTCAGGA-TT-3' (base 6-30) and P_6, 5'-TGGTAGGGATACCTTGTTACGACTTA-3' (base 1491-

1516) (Deng and Hiruki, 1991); F_2, 5'-ACGACTGCTGCTAAGACTGG-3' (base 152-158) and R_2, 5'-TGACGGGCGGTGTGTACAAA-CCCCG-3' (base 1373-1397) (Lee *et al.*, 1993), were synthesized on the basis of 16S rRNA sequence of *Oenothera hookeri* phytoplasma (Lim and Sears, 1989).

Total DNA samples were diluted in sterile deionized water before being subjected to PCR amplifications. Each reaction was performed in a 100 µl mixture with 200 µM dATP, dCTP, dGTP, and dTTP, 0.5 µM primer pair, 2.5 units of Ampli*Taq* DNA polymerase (Promega, Madison, WI). Thirty-five reaction cycles were carried out in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: denaturation 1 min at 94°C, annealing 2 min at 58°C, extension 3 min at 72°C. PCR products were analyzed by electrophoresis in 1% agrose gel followed by staining in ethidium bromide. DNA bands were visualized using a UV transilluminator.

IV.2.4 Nested-PCR

PCR products initially amplified by external primer pairs P1/P6 were diluted in ddH_2O and used as templates for subsequent amplification using the same PCR condition as above with internal primer pairs F2/R2.

IV.2.5 RFLP Analyses of Nested-PCR Products

Nested-PCR products were analyzed by restriction endonuclease digestion. Aliquots about 5 µl were separately digested with each of the following restriction endonuclease according to the instructions of the manufacturer: *Alu*I, *Mse*I, *Rsa*I, *Hpa*II, *Sau*3AI, and *Hha*I (GIBCO BRL, Burlington). The restriction products were resolved on

vertical 5% (w/v) polyacrylamide gels in TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA, pH 8.0). The DNA bands were visualized under UV light after staining with ethidium bromide.

IV.2.6 Sequencing of the Amplified Fragments

DNA fragments about 1.5 kb amplified by external primer pairs P1/P6 were purified using a PCR clean up kit (Boechringer Mannheim Biochemica, Germany). The DNA fragment amplified by primer pair P1/P6 was directly sequenced with *Taq* DNA polymerase. The sequencing primer F2 was labeled as required with 2 pmol [γ-^{33}P]ATP (3000 Ci/mmol) (Amersham) using T_4 polynucleotide kinase as described by Sambrook *et al.* (1989). The sequencing conditions were as follows; in a 10 µl reaction mixture, 1 pmole end labeled primer, 50-100 ng DNA template, 1.25 units of *Taq* DNA polymerase (GIBCO BRL) in 30 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, 30 mM KCl, 0.05% (w/v) gelatin, 20 µM each dATP, dCTP, dTTP, and 7-deaza-dGTP (Boehringer Mannheim), and each 400 µM ddATP, 200 µM ddCTP, 40 µM ddGTP, and 400 µM ddTTP. The mixtures were covered with 30 µl mineral oil. The reaction was carried out for 35 cycles in a thermocycler (Perki-Elmer Cetus, Norwalk, CT) using the following conditions; denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C, extension for 60 s at 72 °C. After amplification, 5 µl of stop solution [95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol] was added to each reaction tube. After denaturation at 90 °C for 5 min, 4 µl of each sequencing product was separated in a 6% (w/v) polyacrylamide/8 M urea sequencing gel.

IV.3 RESULTS

IV.3.1 Amplification of 16S rDNA Sequences Phytoplasmas

Using two sets of primer pairs P1/P6 and R2/F2, DNA fragments about 1.5 kb and 1.2 kb were respectively amplified from all phytoplasma-infected tissues but not from healthy samples. The constant and sharp DNA band was also observed in 1% (w/v) agarose gel after nested-PCR (Fig.IV.1), indicating that the DNA fragments were amplified from phytoplasmas.

IV.3.2 RFLP Analysis

When nested-PCR product (1.2 kb) was digested by restriction endonucleases *Alu*I, *Mse*I, *Rsa*I, *Hpa*II, *Sau*3AI, and *Hha*I respectively, collective RFLP patterns of PaWB isolates from China, Japan, and Korea were identical to one another and very similar to those of AY27, indicating that PaWB isolates from the three different countries are very closely related and belong to group I together with AY27 (Fig.IV.2). Similarly, the identical RFLP profiles with all restriction endonucleases were also obtained using JWB isolates from these three countries, implying that the diseases of jujube witches'-broom in diverse geographic origins are caused by very similar phytoplasma strains.

IV.3.3 Identification of JWB Phytoplasma

After digestion of PCR-amplified DNA fragment (1.2 kb) from phytoplasma isolates with *Alu*I, *Mse*I, *Sau*3AI, and *Hha*I endonucleases, the RFLP patterns of all JWB phytoplasma isolates were very similar to those of elm yellows phytoplasma (Fig.IV.2.A,

B, C, D), but not with *Rsa*I and *Hpa*II (Fig.IV.2.E, F). JWB phytoplasma isolates produced unique RFLP profiles when *Hpa*II endonuclease was used for analysis and produced similar RFLP patterns with CP, PWB and AWB using *Rsa*I. Therefore, JWB phytoplasma strain was identified as a member of the EY group (16Sr V) but different from EY phytoplasma. They should be divided into different subgroups. But JWB phytoplasma isolates from different geographic areas are closely related one another.

IV.3.4 Sequencing Analyses of the Amplified Fragment

The approximately 1.5 kb PCR-amplified fragment was used as a template for direct sequencing by Ampli*Taq* DNA polymerase. The results indicated that the level of 16S rRNA gene sequence homology among PaWB phytoplasma isolates from China, Japan, and Korea was 100% respectively and 99.8% between them and the *Oenothera hookeri* phytoplasma (Fig.IV.3, and 4). In a comparison with the nucleotide sequence of the group I phytoplasmas reported by Namba *et al.* (1993), our nucleotide sequence data of PaWB phytoplasma DNA, including Japanese PaWB isolates, showed a deletion of C at the nucleotide position 1348 of that of group I phytoplasmas. The nucleotide sequences of JWB phytoplasma isolates from the three countries were identical but differ from those of PaWB phytoplasma isolates. The sequence homology between PaWB and JWB phytoplasmas was approximately 94%. These results indicated that witches'-broom diseases of paulownia or of jujube in China, Japan, and Korea was caused by the same or very closely related phytoplasmas respectively, but PaWB and JWB phytoplasmas were genetically distinctive strains.

IV.4 DISCUSSION

For DNA sequencing, several methods have been described including direct sequencing (Wong *et al.*, 1987; Scharf *et al.*, 1986), *in vitro* transcription-based sequencing (Stoflet *et al.*, 1988), asymmetric amplification followed by sequencing (Gyllenstern and Erlich, 1988; Innis *et al.*, 1988), sequencing using linear PCR amplification (Murray, 1989), and coupled amplification and sequencing (Ruano and Kidd, 1991a, b). More recently, a single step procedure for simultaneous amplification and sequencing (SAS) of genomic DNA was reported (Deng *et al.*, 1993). This protocol is simple, fast and reliable for sequencing the target DNA. However, the bands are frequently observed across all four lanes of the sequencing gel since the highly specific SAS conditions are required. Sometimes the sequencing reaction does not develop sufficiently because the very stable secondary structure of template has prevented the DNA polymerase from advancing past the foldback position. In this study, we used a DNA fragment (1.5 kb) which was amplified by external primer pairs as a template for DNA sequencing by *Taq* enzyme using an internal primer. The results obtained were allowed reading of the sequence bases up to 600 bp (Fig.IV.3). To read all sequence bases, two primers were labeled and used for sequencing a DNA fragment from 5'-end and 3'-end respectively. Since primer pairs used in this study are universal primers for phytoplasmas, it is an ideal protocol for 16S rRNA gene sequencing of other phytoplasmas as well.

Analyses of 16S rDNA have been widely used for identification and classification of phytoplasmas. On the basis of 16S rDNA restriction patterns of 52 phytoplasma

isolates, seven major taxonomic groups were established (Schneider, *et al.*, 1993). The validity of these groups was supported by performing a sequence analysis of the 16S rDNA of 17 representative phytoplasmas (Seemüller *et al.*, 1994). Recently, phytoplasmas were suggested to be classified into 11 distinct 16S rRNA groups and 26 subgroups on the basis of RFLP analyses of 16S rDNA fragment and ribosomal protein gene sequences (Lee *et al.*, 1993; Gundersen *et al.*, 1994, 1996) (Fig. I.1, Chapter I). However, the genetic relatedness of JWB or PaWB phytoplasma isolates in diverse geographical areas has not been reported. Our results revealed that JWB phytoplasma isolates from China, Japan, and Korea belongs to the EY group (16Sr V), but distinct from the EY phytoplasma. Therefore, we propose that the members in the group 16Sr V should be divided into two subgroups consisting of subgroup 16Sr V-A and 16Sr V-B. EY and related phytoplasma strains are the members of subgroup 16Sr V-A while strain JWB belongs to subgroup 16Sr V-B. Thus, phytoplasmas in group 16Sr V are more diverse than previously thought.

PaWB phytoplasma belongs to subgroup 16SrI-D together with other aster yellows type strains (Lee *et al.*, 1993). Our sequence data demonstrated that PaWB phytoplasma is closely related to the phytoplasmas of western aster yellows and *Oenothera* phytoplasmas and the PaWB phytoplasma isolates from China, Japan, and Korea share the same 16S rDNA sequences. The similar group identification was also shown on JWB phytoplasmal 16S rRNA genes, indicating that 16S rDNA of phytoplasmas has been highly conserved during their evolution. Cloned DNA probes have also revealed that elm witches'-broom phytoplasma isolates in European and North America are very closely related to each other (Mäurer *et al.*, 1993). All Japanese group I

phytoplasmas including paulownia witches'-broom, tomato yellows, onion yellows and mulberry dwarf share the same 16S rDNA sequences (Namba *et al.*, 1993). In earlier studies, RFLP analysis of 16S rDNA sequences and cloned DNA probes produced from the restriction fragments of 16S rDNA sequences failed to differentiate very closely related phytoplasmas (Lee *et al.*, 1992, 1993; Griffiths *et al.*, 1994). For further study to reveal a finer level of strain differentiation within each phytoplasma group, less conserved gene sequences such as ribosomal protein gene operons should be used for molecular characterization (Gundersen *et al.*, 1996). Alternatively, as shown in our recent work, more sensitive techniques such as DNA heteroduplex mobility assay or/and temperature gradient gel electrophoresis may be used in combination for this purpose (Zhong and Hiruki, 1994; Ceranic-Zagorac and Hiruki, 1996).

The phytoplasma isolates associated with paulownia or jujube witches'-broom in different countries in eastern Asia are very closely related to one another and have the same 16S rDNA sequences respectively in spite of geographical separation. In this respect, it is also interesting to note that PaWB phytoplasma in Japan share the same 16S rDNA sequences with tomato yellows, onion yellows, and mulberry dwarf phytoplasmas, and closely related to *Oenothera* phytoplasma (Namba *et al.*, 1993; Yoshikawa *et al.*, 1994), although PaWB phytoplasma is transmitted by an hemipteran, *Halyomorpha holys* and *H. mista* (Shao *et al.*, 1982; Shiozawa, 1986), in contrast to others that are transmitted by leafhoppers. Further genetic studies are required to clarify mechanisms of vector specificity in understanding epidemiological aspects of these tree diseases.

Fig.IV.1. Nested-PCR amplified 1.2 kb 16S rDNA fragments from DNA preparations from phytoplasma-infected tissues. Lane 1, 100 bp DNA ladder; lane 2, healthy paulownia; lane 3, healthy jujube; lanes 4-7, PaWB isolates from China, Japan1, Japan2, and Korea respectively; lanes 8-10, phytoplasma isolates from China, Japan, and Korea respectively; lane 11, AY27 phytoplasma; lane 12, elm yellows phytoplasam; lane 13, clover proliferation phytoplasma; lane 14, potato witches'-broom phytoplasma; lane 15, alfalfa witches'-broom phytoplasma.

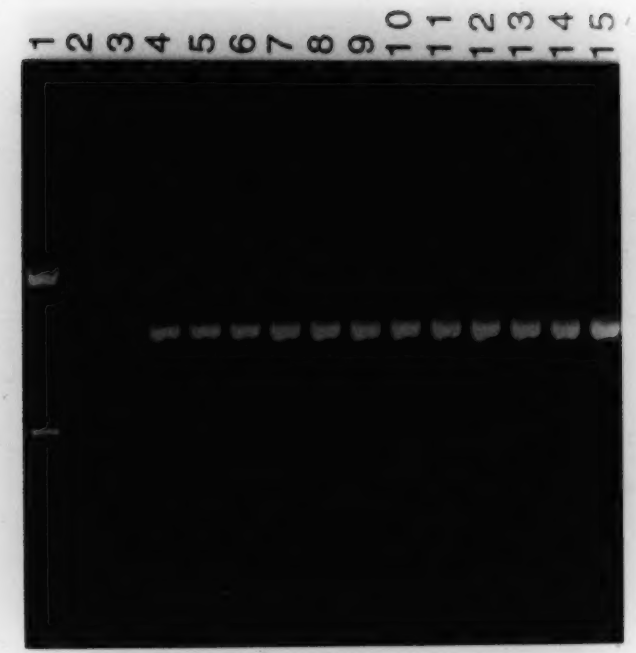

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Fig.IV.2. Restriction fragment length polymorphism analysis of nested-PCR amplified 1.2 kb 16S rDNA fragments using primer pair F2/R2. The PCR products were digested with restriction endonucleases *Alu*I (A), *Hha*I (B), *Mse*I(C), *Sau*3AI (D), *Rsa*I (E), and *Hpa*II(F), and separated by electrophoresis in a 5% (w/v) polyacrylamide gel. Lane 1, 100 bp DNA ladder; lane 2, AY27; lanes 3-6, PaWB from China, Japan1, Japan2, and Korea respectively; lane 7, elm yellows; lanes 8-10, JWB from China, Japan, and Korea respectively; lanes 11, clover proliferation; lane 12, potato witches'-broom; lane 13, alfalfa witches'-broom.

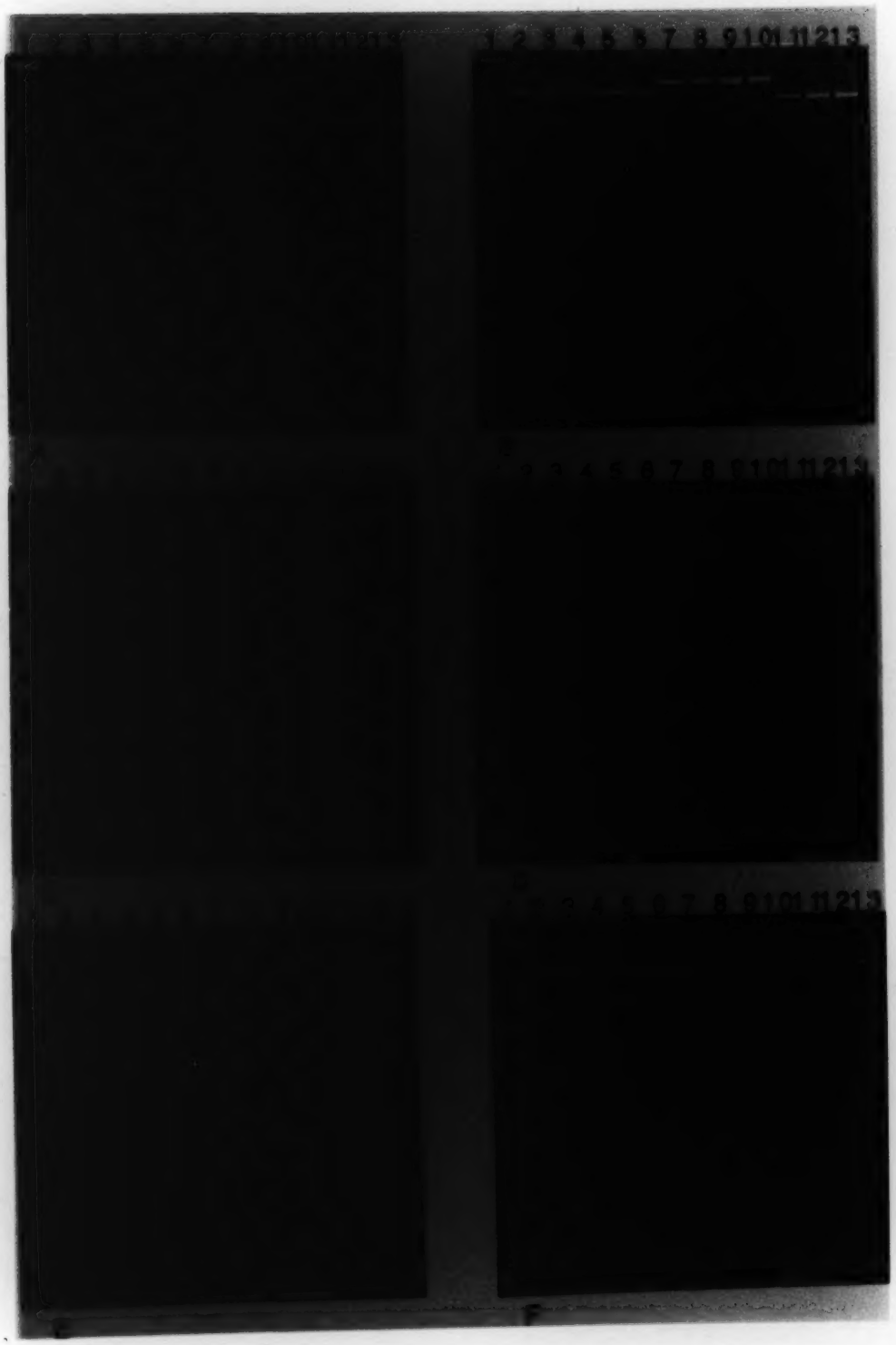


Fig.IV.3. Autoradiograph of a polyacrylamide/urea gel showing sequencing reaction products produced using 1.5 kb DNA fragment amplified from total DNA isolated from paulownia and jujube plants collected in the field which are known to be infected with PaWB and JWB phytoplasmas. The sequence generated using universal primer R_2 is shown for: PaWB phytoplasma isolates from China, Japan, and Korea, JWB phytoplasma isolates from China, Japan, and Korea.

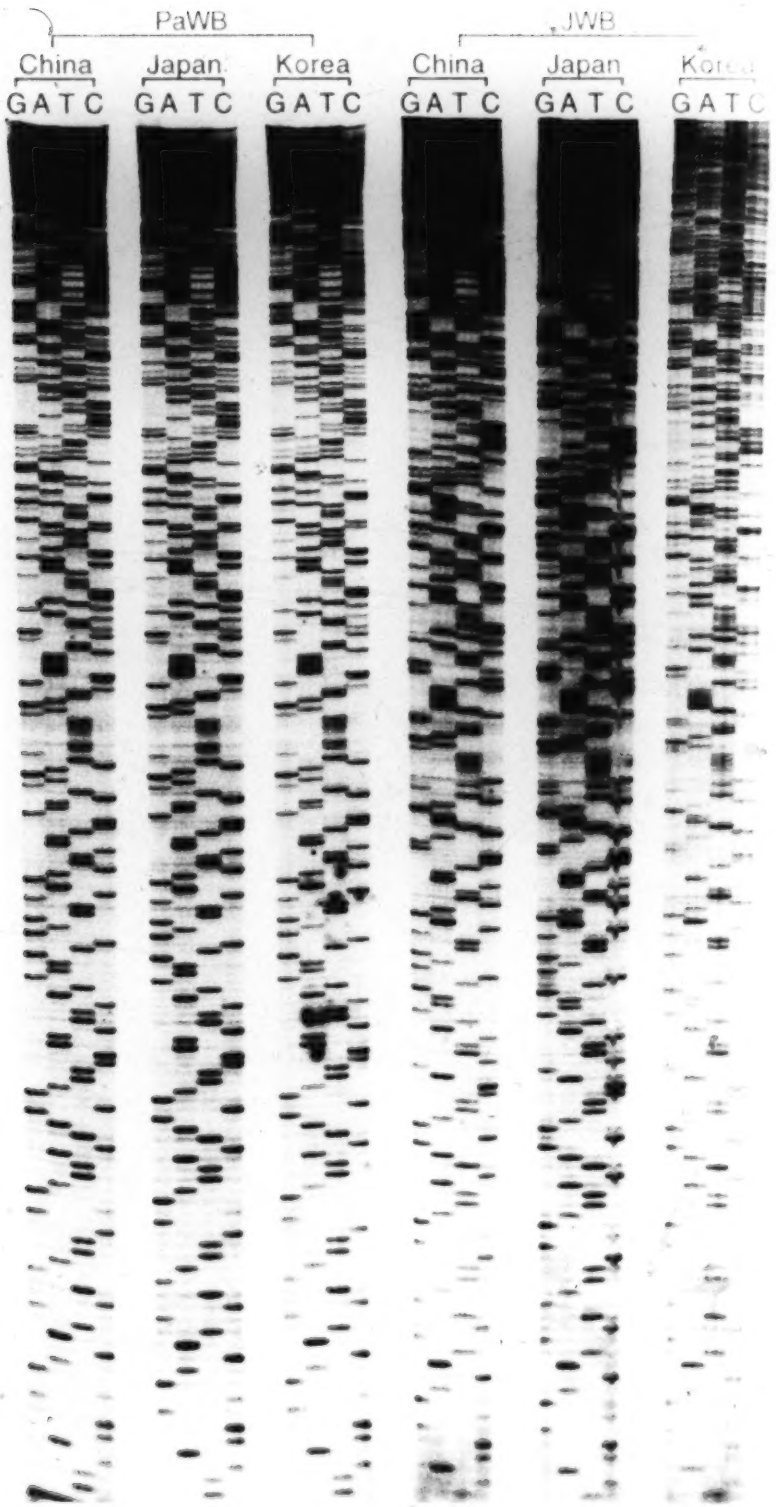
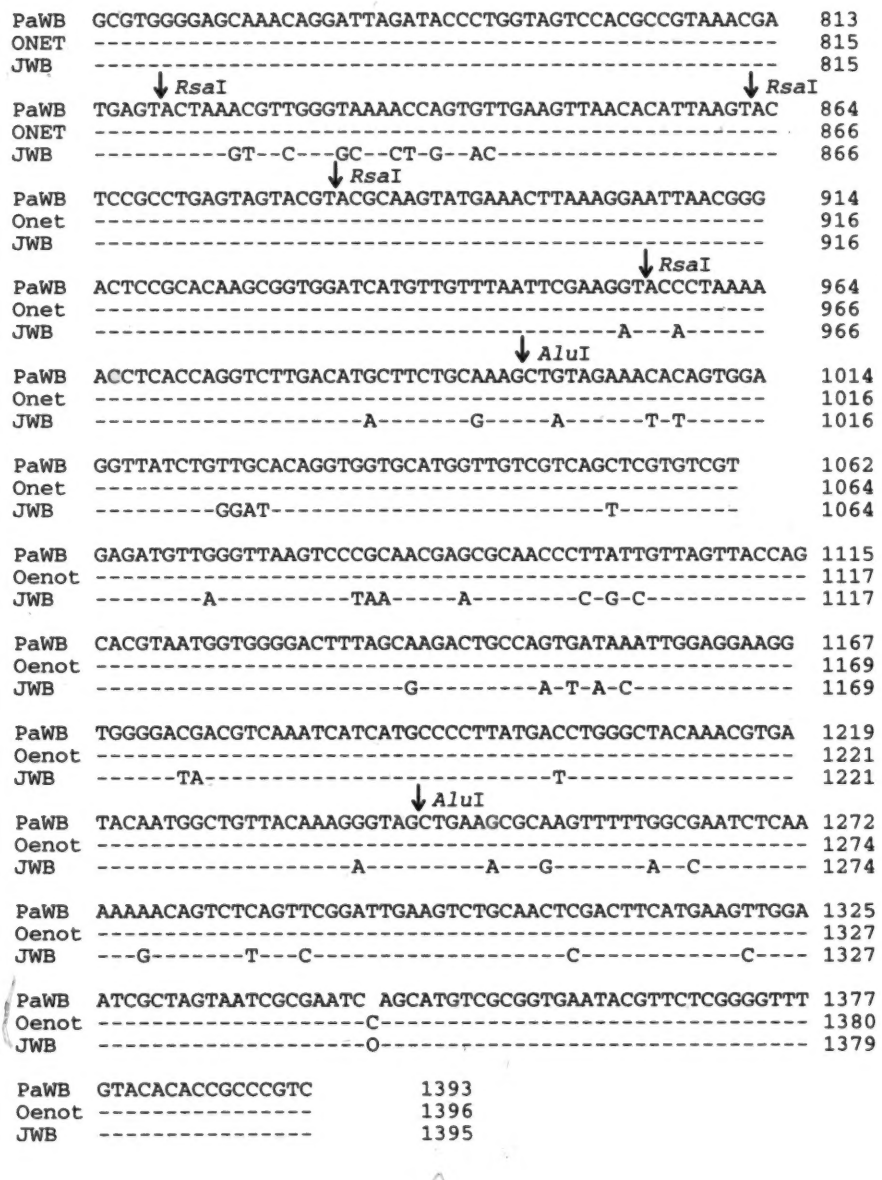

PaWB
JWB
China
Japan
Korea
China
Japan
Korea
G A T C
G A T C
G A T C
G A T C
G A T C
G A T C

Fig.IV.4. Nucleotide sequences of 16S rRNA genes of PaWB and JWB phytoplasmas and corresponding region of those from *Oenothera* phytoplasma. Bases of PaWB and JWB phytoplasmas that are the same as *Oenothera* phytoplasma are shown as dashes.

```
          152
PaWB  ACGACTGCTAAGACTGGATAGGAGACAAGAAGGCATCTTCTTGTTTTTAAAA  203
ONET  ----------------------------------------------------  203
JWB   ----------------------------------T-----------------  203
                                    ↓ AluI
PaWB  GACCTAGCAAT A GGTATGCTTAGGGAGGAGCTTGCGTCACATTAGTTAGTT  254
ONET  -----------O-O----------------------------------------  254
JWB   -----TCTTCGG-G----------AA----------------------------  256

PaWB  GGTGGGGTAAAGGCCTACCAAGACTATGATGTGTAGCCGGGCTGAGAGGTT   305
ONET  ----------------------------------------------------  305
JWB   ---A----------T--------T-------------T--A----------   307

PaWB  GAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGC   356
ONET  ----------------------------------------------------  356
JWB   ----A-----------------------------------------------  358

PaWB  AGTAGGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAACGCCGCGTG   407
ONET  ----------------------------------------------------  407
JWB   ---------------------------------------G----------  409

PaWB  AACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAG AATAAA   457
ONET  ---------------------------------------------G------  458
JWB   --------------------T----------------GAA----A-----T  460
                           ↓ RsaI
PaWB  TGATGGAAAAATCATT CTGACGGTACCTAATGAATAAGCCCCGGCTAACT   507
ONET  ----------------G----------------------------------  509
JWB   A-O--------O-----------T--TTC----------------------  509

PaWB  ATGTGCCAGCAGCCGCTGTAATACATAGGGGGCAAGCGTTATCCGGAATTA   558
ONET  ----------------------------------------------------  560
JWB   ----------------------------------------------------  560

PaWB  TTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCA   609
ONET  ----------------------------------------------------  611
JWB   ----------------------------G------C---AA-T---T-T--  611
                                        ↓ AluI
PaWB  ATGCTCAACATTGTGATGCTATAAAAACTGTTTAGCTAGAGTAAGATAGAG   660
ONET  ----------------------------------------------------  662
JWB   G----T---GC---CT--T----G--T-GTC--GA-------G--------  662

PaWB  GCAAGTGGAATTCCATGTGTAGTGGTAAAATGCGTAAATATATGGAGGAAC   711
ONET  -----C----------------------------------------------  713
JWB   -----C----------------C----------------------------  713

PaWB  ACCAGTAGCGAAGGCGGCTTGCTGGGTCTTTACTGACGCTGAGGCACGAAA   762
ONET  ----------------------------------------------------  764
JWB   -----A----T------------------------------------------  764
```


PaWB GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA 813
ONET 815
JWB 815
RsaI
RsaI
PaWB TGAGTACTAAACGTTGGGTAAAACCAGTGTTGAAGTTAACACATTAAGTAC 864
ONET 866
JWB ----------GT--C---GC--CT-G--AC---------- 866
RsaI
PaWB TCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTAACGGG 914
Onet 916
JWB 916
RsaI
PaWB ACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCTAAAA 964
Onet 966
JWB ----------A---A------ 966
AluI
PaWB ACCTCACCAGGTCTTGACATGCTTCTGCAAAGCTGTAGAAACACAGTGGA 1014
Onet 1016
JWB ----------A-------G-----A------T-T------ 1016
PaWB GGTTATCTGTTGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT 1062
Onet 1064
JWB ---------GGAT----------T--------- 1064
PaWB GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTACCAG 1115
Oenot 1117
JWB --------A----------TAA-----A--------C-G-C---------- 1117
PaWB CACGTAATGGTGGGGACTTTAGCAAGACTGCCAGTGATAAATTGGAGGAAGG 1167
Oenot 1169
JWB ----------G---------A-T-A-C---------- 1169
PaWB TGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGA 1219
Oenot 1221
JWB ------TA----------T---------- 1221
AluI
PaWB TACAATGGCTGTTACAAAGGGTAGCTGAAGCGCAAGTTTTTGGCGAATCTCAA 1272
Oenot 1274
JWB ----------A---------A---G-------A--C-------- 1274
PaWB AAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGA 1325
Oenot 1327
JWB ---G-------T---C----------C----------C---- 1327
PaWB ATCGCTAGTAATCGCGAATC AGCATGTCGCGGTGAATACGTTCTCGGGGTTT 1377
Oenot ----------C---------- 1380
JWB ----------O---------- 1379
PaWB GTACACACCGCCCGTC 1393
Oenot ---------- 1396
JWB ---------- 1395

IV.5 REFERENCES

Ceranic-Zagorac, P., and Hiruki, C. 1996. Comparative molecular studies on aster yellows phytoplasmas. Acta Hort. 432, 266-276.

Chi, L. 1951. An introduction to the study of jujube witches'-broom. Hebei Agr. Forest. 5, 17-19.

Deng, S.J., and Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods 14, 53-61.

Deng, S.J., Forster, R.J., Hiruki, C., and Teather, R.M. 1993. Simultaneous amplification and sequencing of genomic DNA (SAS): sequencing of 16S rRNA genes using total genomic DNA from *Butyrivibrio fibrisolvens*, and detection and genotyping of nonculturable mycoplasma-like organisms directly from total DNA isolated from infected plants. J. Microbiol. Methods 17, 103-113.

Doi, Y., and Asuyama, H. 1981. Paulownia witches'-broom disease. *In*: Mycoplasma Diseases of Trees and Shrubs, p. 135-146, K. Maramorosch and S.P. Taychaudhuri (eds.). Academic Press, New York.

Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. 1967. Mycoplasma or P.T.L. group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches'-broom, aster yellows or paulownia witches'-broom. Ann. Phytopathol. Soc. Japan 33, 259-266.

Griffiths, H.M. Gundersen, D.E., Sinclair, W.A., Lee, I.-M., and Davis, R.E. 1994. Mycoplasmalike organisms from milkweed, goldenrod, and spirea represent two new 16S rRNA subgroups and three new strain subclusters related to X-disease. Can. J. Plant Pathol. 16, 255-260.

Gundersen, D.E., Lee, I.-M., Rehner, S.A., Davis, R.E., and Kingsbury, D.T. 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas); a basis for their classification. J. Bacteriol. 176, 5244-5254.

Gundersen, D.E., Lee, I.-M., Schaff, D.A., Harrison, N.A., Chang, C.J., Davis, R.E., and Kingsbury, D.T. 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int. J. Syst. Bacteriol. 46, 64-75.

Gyllenstern, U.B., and Erlich, H.A. 1988. Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the *HLA-DA* locus. Proc. Natl. Acad. Sci. USA 85, 7652-7656.

Hiruki, C. ed. 1988a. Tree Mycoplasmas and Mycoplasma Diseases. The University of Alberta Press, Edmonton, Alberta.

Hiruki, C. 1988b. Fluorescence microscopy of yellows diseases associated with plant mycoplasma-like organisms. *In*: Mycoplasma Diseases of Crops: Basic and Applied Aspects, p.51-76, K. Maramorosch and S.P. Raychaudhuri (eds), Springer Verlag, New York.

Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A.D. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerse chain reaction-amplified DNA. Proc. Natl. Acad. Sci. USA 85, 9436-9440.

Jin, K.X. 1982. Study on paulownia witches'-broom. *In*: Collected Letters on Paulownia, p.116-126, Chinese Forest Soc. (ed.), Chinese Forestry Publishing Co., Beijing.

Jin, K.X. 1983. Mycoplasma diseases of trees and their control in China. Proc. 6th IUFRO Mycoplamsma Conference, Hiroshima, Japan. (Abst.)

La, Y.J., Pyun, B.H., and Sim, K.J. 1968. Transmission of paulownia witches'-broom virus by tobacco leaf bug *Cyrtopeltis tenuis* Reuter. Korean J. Plant Prot. 5, 1-8.

La, Y.J. 1987. Current status of researches on mycoplasmal diseases of plant in Korea. *In*: Mycoplasma Diseases of Woody Plants, p. 112-128, S.P. Raychaudhuri and N. Rishi (eds), New Delhi.

Kirkpatrick, B., Smart, C., Gardner, S., Gao, J-L., Ahrens, U., Maurer, R., Schneider, B., Lorenz, K.-H., Seemuller, E., Harrison, N., Namba, S., and Daire, X. 1994. Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA space sequences. IOM Letters 3, 228-229.

Lee, I.-M., and Davis, R.E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1, 303-310.

Lee, I.-M., Gundersen, D.E., Davis, R.E., and Chiykowski, L.N. 1992. Identification and analysis of a genomic strain cluster of mycoplasmalike organisms associated with Canadian peach, eastern X-disease, western X-disease, and clover yellow edge. J. Bacteriol. 174, 6694-6698.

Lee, I.-M., Hammond, R.W., Davis, R.E., and Gundersen, D.E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834-842.

Lim, P.O., and Sears, B.B. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionarily distinct from animal mycoplasmas. J. Bacteriol. 171, 5901-5906.

Mäurer, R., Seemüller, E., and Sinclair, W.A. 1993. Genetic relatedness of mycoplasmalike organisms affecting elm, alder, and ash in Europe and North America. Phytopathology 83, 971-976.

Murray, V. 1989. Improved double-stranded DNA sequencing using the linear polymerase chain reaction. Nucleic Acids Res. 17, 8889.

Namba, S., Dyaizu, H., Kato, S., Iwanami, S., and Tsuchizaki, T. 1993. Phytogenetic diversity of phytopathogenic mycoplasmalike organisms. Int. J. Syst. Bacteriol. 43, 461-467.

Ruano, G., and Kidd, K.K. 1991a. Coupled amplification and sequencing of genomic DNA. Proc. Natl. Acad. Sci. USA 88, 2815-2819.

Ruano, G., and Kidd, K.K. 1991b. Genotyping and haplotyping of polymorphisms directly from genomic DNA via coupled amplification and sequencing (CAS). Nucleic Acids Res. 19, 6877-6882.

Sambrook, J., Fritch, E.F., and Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, p. 11.21-11.32, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Scharf, S.J., Horn, G.T., and Erlich, H.A. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233, 1076-1078.

Schneider, B., Ahrens, U., Kirkpatrick, B.C., and Seemüller, E. 1993. Classification of plant-pathogenic mycoplasmalike organisms using restriction-site analysis of PCR-amplified 16S rDNA. J. Gen. Microbiol. 139, 519-527.

Seemüller, E., Schneider, B., Mäurer, R., Ahrens, U., Daire, S., Kison, H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B.B., and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. Int. J. Syst. Bacteriol. 44, 440-446.

Shao, P.H., Huang, J.F., Tung, D.C., Pan, C.H., Shen, C.Y., and Chen, T.Y. 1982. Studies on the insect vector of paulownia witches'-broom I. Transmission By *Halyomorpha halys* (Stal) and electron microscope observation. Shandong Forest. Sci. & Technol. 1, 42-45.

Shiozawa, H. 1986. Transmission of paulownia witches'-broom by stink bugs, *Halyomorpha mista* UHLER. Bull. Res. Lab. Japan Plant Protec. Assoc. 4, 45-50.

Stoflet, E.S., Koeberl, D.D., Sarkar, G., and Sommer, S.S. 1988. Genomic amplification with transcript sequencing. Science 239, 491-494.

Tian, G., and Raychaudhuri, S.P. 1996. Paulownia witches'-broom disease in China: present status. *In*: Forest Trees and Palms: Diseases and Control, p. 227-251, S.P. Raychaudhuri and K. Maramorosch (eds), Science Publishers, Inc. New Hampshire.

Tsai, J.H., Chen, X.Y., Shen, C.Y., and Jin, K.X. 1988. Mycoplasmas and fastidious vascular prokaryotes associated with tree disease in China. *In:* Tree Mycoplasmas and Mycoplasma Diseases, p.69-96, C. Hiruki (ed.), The University of Alberta Press, Alberta.

Wong, C., Dowling, C.E., Saiki, R.K., Higuchi, R.G., Erlich, H.A., and Kazazian, H.H. 1987. Cahracterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 330, 384-386.

Yoshikawa, N. Nakamura, H., Sahashi, N., Kubono, T. Katsube, K. Shoji, T., and Takahashi, T. 1994. Amplification and nucleotide sequences of ribosomal protein and 16S rRNA genes of mycoplasma-like organism associated with paulownia witches'-broom. Ann. Phytopath. Soc. Japan 60, 569-575.

Zhong, Q., and Hiruki, C. 1994. Genetic differentiation of phytoplasma isolates determined by a DNA heteroduplex mobility assay. Proc. Japan Acad. 70B, 127-131.

CHAPTER V

MONARDA YELLOWS, A NEW DISEASE ASSOCIATED WITH A PHYTOPLASMA OF THE ASTER YELLOWS SUBCLADE *

---

* A version of this chapter has been accepted for publication and is in press.

Wang, K, and Hiruki, C. 1997. Plant Pathology

V.1 INTRODUCTION

Monarda (*Monarda fistulosa* L.), commonly known as wild bergamot, is a perennial member of the mint family *Labiatae,* is indigenous to North America (Conners 1967; Scoggan, 1978), and grows in dry thickets, clearings, and borders of woods. Since the early 1980s, monarda has been grown in the prairie region of Canada as a new special crop. It produces high quality geraniol, an essential oil that is used in perfumes and other scented consumer products (Marshall & Chubey, 1983). Monarda is also grown in family gardens as a winter-hardy ornamental as it produces showy, rose-purple, fragrant flowers.

Since 1990, monarda plants showing yellows symptoms have been observed in southern Alberta. The diseased plants showed leaf yellowing, stunting and phyllody. In Alberta, several plant diseases including clover proliferation (CP), potato witches'-broom (PWB), alfalfa witches'-broom, and aster yellows (AY) have been reported to be associated with phytoplasmas causing severe damages to economically important plants (Hiruki & Chen, 1984). So far, their causal agents were identified in two groups of phytoplasmas, AY group and CP group. No previous report was presented about monarda yellows disease.

In this chapter, a new plant disease, monarda yellows (MY), is described and is shown to be associated with a phytoplasma. The results of a comparative molecular study are reported on the basis of PCR and RFLP analyses for characterization of MY phytoplasma in comparison with known strains of phytoplasmas. Evidence is presented to indicate that the MY phytoplasma is a member of the aster yellows subclade and is different from CP and PWB.

V.2 MATERIALS AND METHODS

V.2.1 Plants and Phytoplasma Strains

Infected monarda plants were collected in southern Alberta and maintained in the greenhouse under the same conditions as other plants used in this study. Phytoplasmas associated with aster yellows (AY27), eastern aster yellows (EAY), potato witches'-broom (PWB), and clover proliferation (CP) were maintained in periwinkle. French hydrangea aster yellows (AYHF) and Belgium hydrangea aster yellows (AYHB) in periwinkle were kindly provided by Dr. M.T. Cousin, Versailles, France.

V.2.2 DNA Extraction

Total nucleic acids were isolated from midrib tissues of healthy and infected plants as described previously, treated with DNase-free RNase and then with proteinase K (Chapter II). The final pellets were suspended in 100 µl TE buffer (pH 8.0).

V.2.3 Primer Pairs and PCR Conditions

A universal primer pair P1/P6 was synthesized on the basis of 16S rRNA gene sequence of Mollicutes (Deng & Hiruki, 1991a). A specific DNA fragment approximately 1.5 kb in molecular size was amplified by using the primer pair. Universal primer pair R16F2n/R2, designed by Gundersen *et al.* (1996), was included in this study. The oligonuleotide sequence of these primers and base locations are:

P1, 5'-AAGAGTTTGATCCTGGCTCAGGATT-3' (6-30)

P6, 5'-CGGTAGGGATACCTTGTTACGACTTA-3' (1491-1516)

R16F2n, 5'-GAAACGACTGCTAAGACTGG-3' (149-168)

R16R2, 5'-TGACGGGCG GTGTGTACAAACCCCG-3' (1373-1397)

DNA samples were diluted in sterile deionized water before the PCR assays. The amplification was carried out as previously described (Chapter II). Thermocycling conditions were as follows: denaturation 1 min (5 min for first cycle) at 94°C, annealing at 50°C for 2 min and then extension at 72°C for 3 min (10 min for final cycle). Tubes with the reaction mixture devoid of DNA template were included in each experiment as negative controls. PCR products were analyzed by electrophoresis in 1.0% (w/v) agarose gel, and DNA bands were stained in ethidium bromide and visualized with a UV transilluminator.

V.2.4 Nested-PCR Assays with Two Universal Primer Pairs

PCR products amplified using the universal primer pair P1/P6 were diluted (1/20) in sterile deionized water and used as templates for a subsequent series of 35 PCR cycles in which the universal primer pair R16F2n/R2 was used in the reaction mixture.

V.2.5 RFLP Analyses of PCR Products

A 16S rDNA fragment (1.2 kb) amplified by nested-PCR using primer pair R16F2n/R2 was analyzed by restriction endonuclease digestion. About 5 µl of each PCR product was digested separately with the following restriction enzymes according to the

instructions of the manufacturer: *Alu*I, *Mse*I, *Sau*3AI, *Hpa*II, *Kpn*I and *Rsa*I (GIBCO/BRL, Gaithersburg, MD). The restriction products were then analyzed by electrophoresis through a 5% (w/v) polyacrylamide gel followed by staining in ethidium bromide. DNA bands were then visualized using a UV transilluminator.

V.2.6 Electron microscopy

The procedures for sample preparation and observation for electron microscopy were the same as described previously (Chen & Hiruki, 1977).

V.3 RESULTS

V.3.1 Disease Symptoms

Naturally infected plants showed general stunting with short internodes and a typical yellowing (Fig.V.1). Affected plants produced abnormal leaves smaller and simpler than those in unaffected plants. Bronzing characterized by purplish reddening along leaf margins including tips of the lower leaves appeared toward the end of a growing season. At late stages of disease development, a proliferation of the inflorescence was obvious. The sepals were abnormally enlarged and the virescent petals, retarded in their development, remained small in size. Floral organs were converted to leaflike structures showing a typical phyllody symptoms and consequently failed to produce seeds. The abnormal elongation of pedicels occurred in mildly affected flowers. Occasionally partial, temporary recovery from these symptoms was found, but continued propagation of such healthy looking plants resulted in the development of typical yellows

symptoms. Infected plants eventually lost vigor and winter hardiness. Visual surveys of affected areas indicated an average of up to about 30% infection under field conditions.

V.3.2 Amplification of 16S rDNA Sequences and Nested PCR

Using two universal primer pairs, 16S rDNA fragments (about 1.2 and 1.5 kb) were separately amplified from DNA samples which were extracted from infected monarda, alsike clover and periwinkle. No DNA band was observed when a sample containing only DNA from healthy plants was examined. A 1.2 kb DNA fragment was consistently amplified by primer pair R16F2n/R2 using the 1.5 kb DNA fragment as a template which had been amplified by universal primer pair P1/P6 (Fig.V.2). The results indicated that 16S rDNA fragments were amplified from phytoplasma DNA and monarda witches'-broom was associated with a phytoplasma.

V.3.3 RFLP Analysis of 16S rDNA of Phytoplasmas

After digestion of the amplified ribosomal DNA by nested-PCR with endonucleases, *Alu*I, *Hpa*II, *Kpn*I, *Rsa*I, *Mse*I, and *Sau*3AI, respective digestion products were compared. The restriction profiles of ribosomal DNA of MY phytoplasma were similar to those of AY27, EAY, AYHF, and AYHB phytoplasmas, but were different from those of CP and PWB. The results showed that MY phytoplasma belongs to the aster yellows group together with AY27, EAY, AYHF, and AYHB phytoplasmas, while CP and PWB phytoplasmas belong to the CP group.

V.3.4 Electron Microscopy

Examination of ultrathin sections of a midrib tissue from a yellows-affected monarda leaf revealed numerous phytoplasma bodies in the sieve tubes. These bodies are typically pleomorphic, mostly measuring 200 to 400 nm in size, bounded by a unit membrane, and lacking cell walls (data not shown). These bodies were absent in the sieve tubes of a comparable healthy plant

V.4 DISCUSSION

Recently, the 16S ribosomal RNA (rRNA) genes have been used as a taxonomic basis to classify both culturable and unculturable bacteria into several different species (Bascunana *et al.*, 1994; Combet-Blanc *et al.*, 1995; Gauthier *et al.*, 1995; Ochi 1995; Rogall, *et al.*, 1990). The approach, in which the highly conserved genes such as 16S rRNA genes or ribosomal protein genes were analyzed by restriction fragment length polymorphism (RFLP), has been proved to be useful for classifying unculturable phytoplasmas (Davis & Lee, 1993; Deng & Hiruki, 1991a, b; Gundersen *et al.*, 1994, 1996; Lee *et al.*, 1993; Namba *et al.*,1993; Schneider *et al.*, 1993). On the basis of RFLP analyses of PCR-amplified 16S rDNA or ribosomal protein (rp) genes, a total of 11 distinct phytoplasma subclades have been established (Gundersen *et al.*, 1994, 1996; Lee *et al.*, 1993). Some key restriction sites which reflect a unique sequence(s) or signatures were identified and demonstrated to be useful in classifying a phytoplasma group or subgroup (Gundersen *et al.*, 1996). Among them, the presence of a *Kpn*I site (around base pair 920) was unique to the members of group I (aster yellows and related phytoplasmas). Hence, the restriction enzyme *Kpn*I was particularly useful to differentiate the AY group

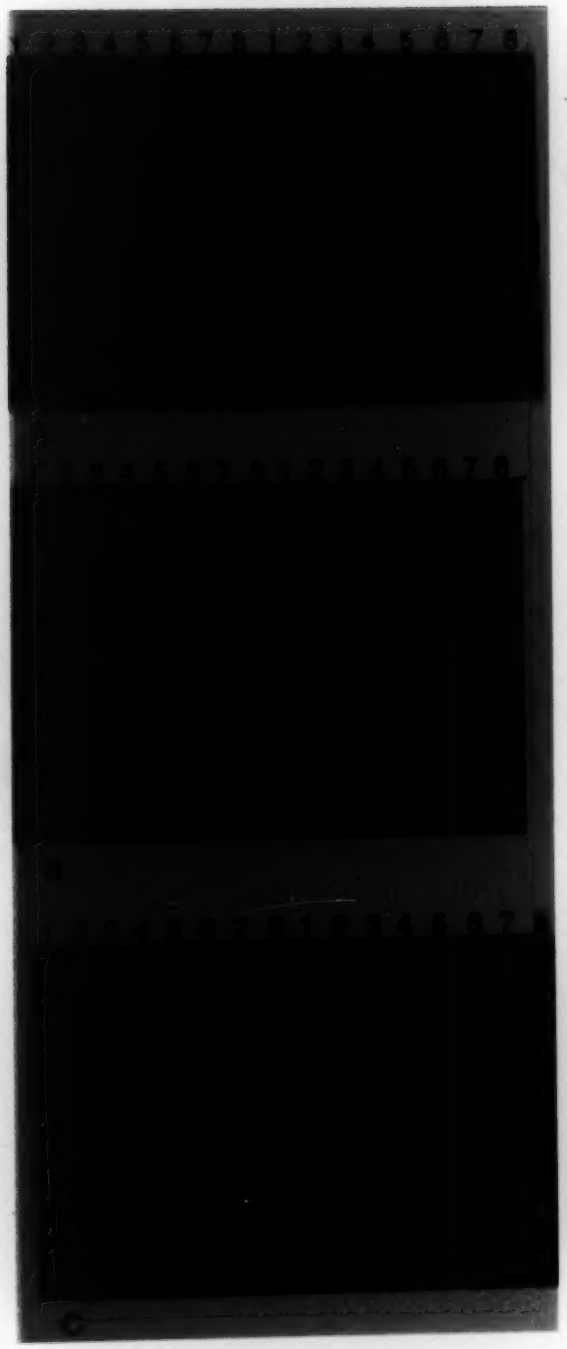
from other groups. *Mse*I, *Alu*I, *Hpa*II and *Hha*I were needed for differentiation among members of group I (Lee *et al.*, 1993). In our study, we also used these restriction enzymes to identify MY phytoplasma. The RFLP patterns of some phytoplasmas used in this study were in agreement with the previous studies on the AY group phytoplasmas (Gundersen *et al.*, 1994, 1996; Lee *et al.*, 1993). This is the first report of the association of monarda yellows with a phytoplasma which belongs to the AY subclade, namely 16SrI-A subgroup, on the basis of RFLP analyses of 16S rDNA sequences.

Fig. 1. The symptoms of monarda yellows showing small leaves, leaf yellowing, and proliferation (A) compared with a healthy plant (B).

Fig.V.2. 16S rDNA fragment (1.2 kb) of phytoplasmas amplified by nested-PCR from various phytoplasma strains. Line 1, 100 bp DNA ladder; Lane 2, healthy monorda; line 3 to 9, MY, AY27, EAY, AYHF, AYHB, CP, PWB.

1 2 3 4 5 6 7 8 9

Fig.V.3.A, B, & C. RFLP profiles of 16S rDNA amplified by nested-PCR from representative phytoplasma strains. A, *Alu*I and *Rsa*I; B, *Mse*I and *Kpn*I; C, *Hpa*II and *Sau*3AI. Line 1, 100 bp DNA ladder; line 2 to 8, MY, AY27, EAY, AYHF, AYHB, CP, PWB.



V.5 REFERENCES

Bascunana, C.R., Mattson, J.G., Bolske, G., and Johansson K.-K. 1994. Characterization of the 16S rRNA gene from *Mycoplasma* sp. strain F38 and development of an identification system based on PCR. J. Bacteriol. 176, 2577-2586.

Chen, M.H., and Hiruki, C. 1977. Effect of dark treatment on the ultrastructure of the aster yellows agent *in situ*. Phytopathology 67, 321-324.

Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B.K.C., Dwivedi, P.P., Pot, B., Prensier, G., and Garcia J.-L. 1995. *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. Int. J. Syst. Bacteriol. 45, 9-16.

Conners, I.L. 1967. An annotated Index of Plant Diseases in Canada. Canada Department of Agriculture. Publication 1251, 381.

Davis, R.E., and Lee, I.-M. 1993. Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology 83, 1008-1011.

Deng, S., and Hiruki, C. 1991a. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. J. Microbiol. Methods 14, 53-61.

Deng, S., and Hiruki, C. 1991b. Genetic relatedness between two non-culturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81, 1475-1479.

Gauthier, G., Lafay, B., Tuimy, R., Breittmayer, V., Nicolas, J.L., Gauthier, M., and Christen, R. 1995. Small-subunit rRNA sequences and whole DNA relatedness concur for reassignment of *Pasteurella piscicida* (Snieszko *et al.*) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. Nov. Int. J. Syst. Bacteriol. 45, 139-144.

Gundersen, D.E., Lee, I.-M., Rehner, S.A., Davis, R.E., and Kingsbury, D.T. 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. J. Bacterol. 176, 5244-5254.

Gundersen, D.E., Lee, I.M., Schaff, D.A., Harrison, N.A., Chang, C.J., Davis, R.E., and Kingsbury, D.T. 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int. J. Syst. Bacteriol. 46, 64-75.

Gundersen, D.E., and Lee, I.-M. 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologie Mediterranea 35, 144-151.

Hiruki, C., and Chen, M.H. 1984. Plant mycoplasma disease occurring in Alberta. (Abstr.) Proc. IUFRO Mycoplasma Conference, the Seventh p.7.

Lee, I.-M,. Hammond, R.W., Davis, R.E., and Gundersen, D.E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834-842.

Marshall, H.H., and Chubey, B.B. 1983. Monarda for geraniol production. Agriculture Canada, Canadex 268, 10.

Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H., and Tsuchizaki, T. 1993. Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. Phytopathology 83, 786-791.

Ochi, K. 1995. Amino acid sequence analysis of ribosomal protein AT-L30 from members of the family *Pseudonocardiaceae.* Int. J. Syst. Bacteriol. 45, 110-115.

Rogall, R., Wolters, J., Flohr, T., and Bottger, E.C. 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium.* Int. J. Syst. Bacteriol. 40, 323-330.

Schneider, B., Ahrens, U., Kirkpatrick, B.C. and Seemüller, E. 1993. Classification of plant pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. J. General Microbiol. 139, 519-527.

Scoggan, H.J. 1978. The Flora of Canada Part 4, p.1312-1313. National Museums of Canada, Ottawa.

CHAPTER VI

PCR (POLYMERASE CHAIN REACTION)-BASED SELECTION OF PHYTOPLASMA-FREE CLONES OF PAULOWNIA TISSUE CULTURE AFTER HEAT TREATMENT OF WITCHES'-BROOM *

---

* A version of this chapter has been published. Wang, K., and Hiruki, C. 1996. Proc. Japan Acad. 72B: 44-47.

VI.1 INTRODUCTION

Paulownia witches'-broom (PaWB) caused by a phytoplasma is one of the most serious diseases to affect paulownia trees. No effective chemotherapy is available to cure PaWB disease at present, due to the inability thus far to isolate phytoplasmas in pure culture and the lack of proper understanding of their properties. Although tetracyclines suppress the development of symptoms and even temporarily eliminate the symptoms, the pathogen still remains in the treated trees, and the symptoms reappear later after the chemical treatments are discontinued (Jin, 1982). To maintain their suppressive effects, the chemical treatments must be repeated at least once a year and must be accompanied by thorough vector control (Raju and Nyland, 1988).

While the presence of insect vectors has been reported for PaWB, rapid disease spread in the field is promoted by vegetative propagation also (Tian and Raychaudhuri, 1996). It is extremely important to use phytoplasma-free materials when propagating paulownia seedlings. Heat-treatment is an effective way against both plant viruses and phytoplasmas of many plant species including fruit trees (Morel, 1964), sugar cane (Quak, 1966), and cassava (Kartha and Gamborg, 1975). In the tissue-cultured meristem, where phytoplasma occurs at low concentrations (Caudwell *et al.*, 1990), the combination of heat-treatment and meristem culture was found to increase the possibility of obtaining phytoplasma-free plants (Pierik, 1987). *In situ,* plant viruses or phytoplasmas could be reduced to low concentrations (Sears and Klomparens, 1989; Ducrocquet *et al.*, 1986), or inactivated (Shukla and Singh, 1990) or eliminated (Lenz *et al.*, 1983; Dai, 1991) by heat treatment.

Since phytoplasmas cannot yet be cultured *in vitro*, the conventional phytoplasma detection methods are not practically effective for rapidly and accurately determining whether the treated tissue cultures are free of phytoplasmas or not. Recently, the polymerase chain reaction (PCR) has been developed and allows the most sensitive and rapid detection of phytoplasmas in infected plants (Deng and Hiruki, 1990, 1991a, b; Schaff *et al.*, 1992; Ahrens and Seemüller, 1992; Namba *et al.*, 1993). The objective of this chapter was to obtain phytoplasma-free plantlets from meristem cultures by PCR-based screening after heat treatment of infected paulownia tissue cultures.

VI.2 MATERIALS AND METHODS

VI.2.1 Sources of Healthy and Phytoplasma-infected Paulownia Tissue Cultures

For phytoplasma-infected paulownia tissue culture, shoots 3 to 4 cm long were excised from a paulownia plant showing typical symptoms of PaWB and surface-sterilized by soaking in 75% (v/v) ethanol for 30 seconds, washed in sterile distilled water, then transferred to 1% (w/v) sodium hypochlorite for 10 to 15 min. The shoots were then rinsed three times in sterile distilled water. The shoots 4 to 5 mm of three to four explants were aseptically placed in MS agar medium without hormones (Murashige and Skoog, 1962). The tissue cultures were maintained in a growth chamber at 25°C under artificial illumination (30 $\mu mol.m^{-2}.s^{-1}$) with a 16-h photoperiod. Healthy paulownia plants were started from seeds. The healthy and diseased paulownia tissue cultures were subcultured to fresh medium at intervals of 1 to 2 months by cutting the tissue cultures in 1-cm-long segments.

VI.2.3 Heat Treatment and Meristem Culture

The shoots with two pairs of axillary buds which were cut from the phytoplasma-infected paulownia tissue cultures were aseptically transferred to fresh MS medium and kept in growth chambers at 35°C at a 16-hr day length with a light intensity of 30 μmol. $m^{-2}.s^{-1}$. The healthy tissue cultures were maintained under the same conditions as those for the diseased tissue cultures. After heat treatment for five weeks, the meristems of shoots were excised from treated tissue cultures and asceptically placed in fresh MS rooting medium containing kinetin (1 mg/l) and α-naphthaleneacetic acid (NAA, 0.1 mg/l) at 25°C.

VI.2.3 DNA Isolation

Total DNAs were isolated from healthy control, phytoplasma-infected and heat treated paulownia tissue cultures by a modified procedure of Namba *et al.* (Namba *et al.*, 1993). The tissues were pulverized in liquid nitrogen with a mortar and pestle. Each ground tissue sample was homogenized in 2.5 ml of DNA extraction buffer [2.5 M NaCl, 0.5% (W/V) PVP-10 (polyvinylpyrrolidone-10), 1% (W/V) cetavlon (hexadecyltrimetlyl-ammonium bromide), 0.5 M Tris-HCl (pH 8.0), 0.2% 2-mercaptoethanol]. The samples were incubated at 65°C for 30 min and then centrifuged at 10,000 *g* for 5 min. The supernatant was mixed with 1 vol. of chloroform-isoamyl alcohol (24:1). After centrifugation at 12,000 *g* for 10 min, the DNA was precipitated by adding 0.7 vol. of isopropanol to the aqueous phase. The final pellets were washed with 70% (v/v) ethanol, dried under vacuum and suspended in 1 ml TE buffer (pH 8.0).

VI.2.4 PCR Detection

Two universal oligonucleotide primers, R16F2 and R16R2 (Lee *et al.*, 1993), which amplify a DNA fragment of about 1.2kb were chosen for this study. For the PCR, total nucleic acid samples were diluted in sterile distilled water as previously described (Chapter II). The total 100 µl reaction mixture contained 50-100 ng of test DNA preparations, PCR reaction buffer (1x 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% (v/v) Triton X-100), 2.5 mM MgCl, 2.5 U of *Taq* polymerase (Promega, Medison, WI), 200 µM four dNTPs and 1 µM each primer. The mixture was covered with 30 µl of mineral oil. Thirty-five PCR cycles were carried out in an automated Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). Cycle conditions were 1 min (5 min for the first cycle) of denaturation at 94°C, 2 min of annealing at 50°C, 3 min (10 min in the final cycle) of primer extension at 72°C. After amplification, a 10-µl aliquot of PCR product from each sample was analyzed by electrophoresis in 1.0% (w/v) agarose gel and visualized by staining with ethidium bromide and by a UV transilluminator.

VI.3 RESULTS AND DISCUSSION

After a 4-week incubation at 25°C, the tissue cultures from phytoplasma-infected paulownia shoots showed the typical phytoplasma symptoms such as small leaves, chlorosis, stunted growth, and abnormally activated growth of axillary buds (Fig.VI.1). Through a heat treatment of the diseased tissue cultures for 5 weeks at 35°C followed by meristem culture at 25°C, the subsequent plantlets appeared healthy with newly enlarged

leaves and elongated internodes. No symptoms of witches'-broom appeared in a series of subcultured clonal plantlets (Fig.VI.2).

The TableVI.1 shows that 94% of the treated tissue cultures were found free of phytoplasma by PCR. The remaining 6% showed a faint DNA band which was amplified from the total DNA, indicating that the phytoplasma in the treated tissues occurred at very low concentrations. The prominent and disease-specific band was observed in 1% (w/v) agarose gels after amplification from all the diseased tissues (Fig.VI.3). The results indicated that the phytoplasma-free paulownia tissue cultures were obtained by PCR-based selection of meristem cultures after the heat treatment.

A currently used paulownia propagation procedure mainly depends on the use of paulownia roots (Tian and Raychaudhuri, 1996). It was found that high rates of disease incidence of PaWB were found in paulownia seedlings raised from roots (Jin, 1980), which contributed to recent rapid increases in the incidence of PaWB since most paulownia roots carry phytoplasma (Tian and Raychaudhuri, 1996). PaWB is mainly spread by vegetatively propagated seedlings and no effective methods are available for control. Therefore, the production of phytoplasma-free paulownia plants is the first, essential step for controlling PaWB. This study provides an effective method for the production of healthy paulownia tissue cultures from which phytoplasma-free paulownia plantations can be established by combining insect control measures.

It was reported that when the susceptible paulownia species in seriously PaWB-affected areas of Henan in the central region of China were transplanted to Sichuan in the south of China, no witches'-broom symptoms were recorded later (Jin, 1980). This suggests that paulownia witches'-broom phytoplasma may had been inactivated or the

disease was masked or both while growing at prevailing higher temperature. The fact that the meristem is nearly free of phytoplasma (Pierik, 1987) apparently contributes to the production of phytoplasma-free paulownia tissue cultures. In addition, the absence of vascular elements in the meristem may effectively hinder the transport of phytoplasma within a developing plant (Kartha and Gamborg, 1975).

The phytoplasmas occur only in the phloem tissues of infected plant hosts. The total DNA from apple proliferation-diseased apple phloem contained about 2% phytoplasma DNA (Kollar *et al.*, 1990). Kirkpatrick's results (Kirkpatrick, 1989) showed that phytoplasma DNA is less than 0.1% (w/v) of the total DNA in the extracts from woody hosts exhibiting disease symptoms. The phytoplasma titer in asymptomatic infected plants isusually very low. PCR serves as the most sensitive method for the detection of phytoplasmas, since as little as 16pg total DNA can be detected by this procedure (Chapter II). Therefore, through screening by PCR, the reliability of establishing phytoplasma-free paulownia tissue cultures by means of heat treatment and meristem culture is strengthened.

Fig. 1. Phytoplasma-infected paulownia tissue culture maintained in a growth chamber for 4 weeks at 25°C showing typical phytoplasma symptoms such as little leaves, chlorosis and abnormally activated axillary buds.

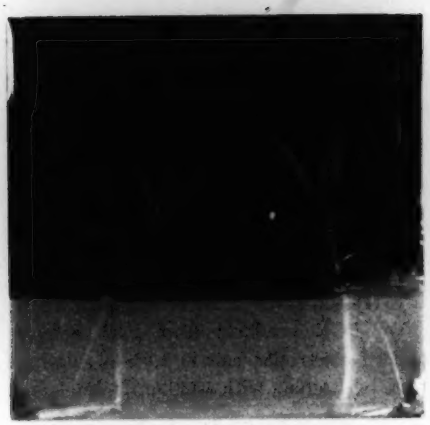


Fig. 2. Paulownia meristem culture maintained at 25°C was heat-treated for 5 weeks at 35°C. The plantlets appeared healthy thereafter, and were found to be phytoplasma-free.

Fig. VI.3. Polymerase chain reaction (PCR) detection of phytoplasma in heat-treatment and untreated PaWB-affected paulownia tissue cultures. Lane 1, DNA marker; lane 2, healthy paulownia culture (control); lanes 3-8, paulownia meristem culture, heat-treated tissues for 5 weeks at 35°C, lanes 3, 4, 6, 7, 8 are phytoplasma-free, lane 5 shows phytoplasma DNA at a low concentration; lanes 9-12, phytoplasma-infected paulownia tissue culture.

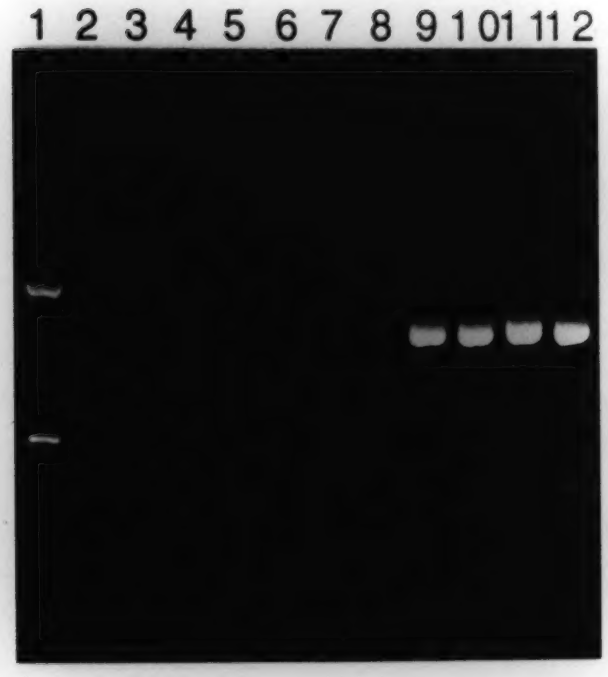

1 2 3 4 5 6 7 8 9 10 11 12

Table 1. The number of phytoplasma-free clones of paulownia tissue cultures obtained through PCR-based screening after heat treatment of witches'-broom

Temperature	Number of meristem	PCR-based screening	
	Tested	Diseased plants	Healthy plants
25°C (diseased)	30	30	0
35°C (diseased)	33	2	31
25 °C (healthy)	15	0	15
35 °C (healthy)	15	0	15

VI.4 REFERENCES

Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82, 828-832.

Caudwell, A., Boudon-Padieu, E., Lherminier, J., Schwartz, Y., and Meignoz, P. 1990. Current spread of the graepvine yellows and characterization methods for the MLO pathogen. Zentralbl. Bacteriol. Suppl. 20, 916-918.

Dai, L. 1991. The preliminary study on eliminating viruses from apple trees. Fruit Tree Sci. 8, 7-12.

Deng, S., and Hiruki, C. 1990. Enhanced detection of a plant pathogenic mycoplasma-like organisms by polymerase chain reaction. Proc. Japan Acad. 66B, 140-144.

Deng, S., and Hiruki, C. 1991a. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81, 1475-1479.

Deng, S., and Hiruki, C. 1991b. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods 14, 53-61.

Ducrocquet, J.P., Dosba, F., Lansac, M., and Mazy, K. 1986. Effect of temperature on symptom express of apple proliferation. Agronomie 6, 897-903.

Jin, K.X. 1980. Studies on paulownia witches'-broom. Chinese J. Plant Protec. 7, 177-182.

Jin, K.X. 1982. Studies on control of paulownia witches'-broom. *In*: Collected Letters on Paulownia, p.116-126, Chinese Forest Soc. (ed.), Chinese Forestry Publishing Co., Beijing.

Kartha, K.K., and Gamborg, O.L. 1975. Elimination of cassava mosaic disease by meristem culture. Phytopathology 65, 826-828.

Kirkpatrick, B.C. 1989. Strategies for characterizing plant pathogenic mycoplasmalike organisms and their effects on plants. *In*: Plant-Microbe Interactions, Molecular and Genetic Perspectives, vol. 3, p. 241-293, T. Kosuge and E.W. Nester (ed.), New York, McGraw-Hill.

Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and bové J.M. 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Phytopathology 80, 233-237.

Lee, I.-M. Hammond, R.W., Davis, R.E., and Gundersen, D.E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83, 834-842.

Lenz, F., Baumann, G., and Kornkamhaeng, P. 1983. High temperature treatment of prunus avium for virus elimination. Phytopathol. Z. 106, 373-375.

Morel, G. 1964. Regeneration des varieties virosees par la culture des meritemes apicaux. Rev. Hort., Paris 136, 733-740.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 474-497.

Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H., and Tsuchizaki, T. 1993. Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. Phytopathology 83, 786-791.

Pierik, R.L.M. 1987. Production of disease-free plants. *In*: *In Vitro* Culture of Higher Plants, p.169-181, R.L.M. Pierik (ed.), Dordrencht, The Netherlands.

Quak, F. 1966. Meristeem cultuur nes middel ter verkrijging van virusvrije planten. Landbouwk. Tijdschr. 78, 301-305.

Raju, B.C., and Nyland, G. 1988. Chemotherapy of mycoplasma diseases. In: Tree Mycoplasmas and Mycoplasma Diseases, p. 207-216, C. Hiruki (ed.), The University of Albeta Press, Edmonton.

Schaff, D.A., Lee, I.-M., and Davis, R.E. 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reaction. Biochem. Biophys. Res. Commun. 186, 1503-1509.

Shukla, U.S., Singh, V. 1990. Inactivation of GSD MLOs in diseased setts by heat treatment. Indian Phytopathol. 43, 561-562.

Sears, B.B., and Klomparens, K.L. 1989. Leaf tip cultures of the evening primrose allow stable, aseptic culture of mycoplasmalike organism. Can. J. Plant Pathol. 11, 343-348.

Tian, G., and Raychaudhuri, S.P. 1996. Paulownia witches'-broom disease in China: present status. *In*: Forest Trees and Palms, Diseases and Control. p, 227-251, S.P. Raychaudhuri and K. Maramorosch (eds.), Science Publishers, Inc. New Hampshire.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

VII.1 GENERAL DISCUSSION

Since the first successful application of polymerase chain reaction (PCR) to the study of phytoplasmas (Deng and Hiruki, 1990a), phytoplasma DNA samples from world-wide collections of phytoplasma isolates have been used successfully as templates for PCR amplification (Ahren *et al.*, 1992, 1993; Schaff *et al.*, 1992; Chen *et al.*, 1993; Lee *et al.*, 1993a, b; Gundersen *et al.*, 1994, 1996). A previous study using nucleic acids extracted from infected woody plants suggested that the minimum amount of DNA required for PCR amplification in 40 cycles was only 170 pg (Ahrens and Seemüller, 1992).Our PCR study in 45 cycles showed that as low as 16 pg of total DNA from infected paulownia plant was sufficient for detection of phytoplasmas in the original host plants by PCR (Chapter II). This has made the PCR technique practical for diagnosis of phytoplasma diseases in the field.

The maintenance of pathogens is a prerequisite for future studies of comparisons of field variants of phytoplasma in nature. The most important aspect on the maintenance of pathogens is that the maintained isolates are genetically identical to the original ones. Chapter II also revealed that micropropagation of the infected host plants was very useful for maintenance of phytoplasmas *in vivo*. Furthermore, it was found that the concentration of phytoplasmas in tissue culture was significantly higher than that in plant grown under greenhouse conditions. The phytoplasma-infected tissue culture exhibited typical symptoms of witches'-broom, small leaves with large stipules and stunted growth (Chapter II and VI). Similar recent studies have reported the successful maintenance of apple-proliferation (AP) phytoplasmas. AP phytoplasma maintained in tissue culture was

genetically identical to that in natural host plant even after 10 years of *in vitro* micropropagation (Jarausch *et al.*, 1996).

Chapter III reported a new, simple and economical method for preservation of phytoplasma DNA by microwave treatment. After the microwave heat treatment of phytoplasma-infected paulownia tissue cultures, midribs, or petioles from 1 to 20 min., phytoplasma DNA was extracted from all of the fresh tissues, all of the microwave-treated tissue cultures, but only from microwave-treated midribs and petioles up to 14 min. RFLP analysis of PCR amplified DNA fragments and 16S rDNA sequence data revealed no genetic difference in the phytoplasma DNA between microwave-treated tissues and fresh material.

The optimum treatment time for microwave dehydration of the tissues was from 6 to 10 min. In addition, DNA was not extracted from air-dried infected paulownia tissues probably due to some enzyme reactions, such as polyphenol oxidase, the peroxidase and corresponding isoenzymes etc., maybe damaging the intactness of the structure of phytoplasma DNA.

Information on the genetic relatedness of phytoplasma isolates is highly useful for developing effective disease control measures. PaWB and JWB diseases are prevalent in Asia including China, Japan, and Korea. However, until now no one has studied the genetic relatedness of their pathogens from these countries. This thesis reports for the first time, the genetic relatedness of phytoplasma isolates occurring in paulownia and jujube from different countries, as revealed by the analyses of their 16S rDNA sequence (Chapter VI). The results indicated that PaWB phytoplasma isolates in the three countries were very closely related to each other but were distinctly different from JWB

phytoplasma isolates which also showed to be very similar among JWB phytoplasma isolates. Many previous studies of the etiology of phytoplasmal diseases, using various molecular biological techniques such as monoclonal antibodies, cloned DNA probes, RFLP analyses of PCR amplified 16S rDNA fragment etc. have provided similar conclusions. For example, Mäurer *et al.*, (1993) demonstrated by nucleic hybridization that European elm witches'-broom and North American elm yellows, as well as European alder yellows were caused by closely related phytoplasmas. RFLP analyses and DNA hybridization have revealed that all ash yellows occurring in 19 contiguous states and two Canadian provinces from Montana and Nebraska eastward to New England were caused by very similar phytoplasmas (Sinclair *et al.*, 1996). Cloned DNA probes derived from a periwinkle-maintained isolate of elm yellows revealed that the EY isolates from the United States were very similar to those in Europe (Lee *et al.*, 1993a). These results have suggested that phytoplasmas associated with single-infection plant diseases from diverse geographical locations are very closely related to each other, i.e. sharing very high nucleotide sequence homology with one another. On the other hand, some mixed infections occurring in certain phytoplasma diseases, such as grapevine yellows, exhibit at least three distinct phytoplasma groups were found and the pathogens vary with different locations (Prince *et al.*, 1993; Alma *et al.*, 1996).

Although more than fifty phytoplasma isolates have been identified by a variety of molecular techniques and have been classified into eleven distinct phytoplasma 16S rRNA groups and more than twenty-five subgroups on the basis of RFLP analyses of PCR-amplified 16S rRNA gene and 16S ribosomal protein gene sequences (Lee *et al.*, 1993b; Gundersen *et al.*, 1994, 1996), only one JWB phytoplasma isolate from China was

identified as a member of 16S rRNA group V (EY and related phytoplasmas) (Kirkpatrick *et al.* 1994). Through comparative molecular studies with other known strains of phytoplasmas, JWB phytoplasma isolates from China, Korea and Japan were found to be in the same group as EY phytoplamsa but different from EY phytoplasma on RFLP profiles.

Chapter VI has also provided important evidence for the highly conserved nature of phytoplasmal 16S rRNA genes. PaWB phytoplasma isolates from different countries share the same 16S rRNA gene sequences. The same results were obtained for JWB phytoplasma isolates which also had the same 16S rRNA gene sequences among JWB phytoplasma isolates. Namba *et al.*, (1993), using specific primers, concluded that several Japanese group I phytoplasmas have the same 16S rRNA sequences. To reveal a finer level of differentiation among the strains in each phytoplasma group, less conserved gene sequences such as ribosomal protein gene operon is recommended (Gundersen *et al.*, 1996).

For DNA sequencing, several techniques have been developed since application of PCR. Recently, a single step procedure called simultaneous amplification and sequencing was described for direct genomic DNA sequencing (Deng *et al.*, 1993). However, heavy compression frequently occurs with this procedure, although it is simple and fast for sequencing the target DNA. A sequencing method described in Chapter IV is capable of overcoming the above-mentioned problems. External primers were used to amplify longer fragment as a template and the internal primer for DNA sequencing by *Taq* enzyme. The results yielded up to 600 bp of discernible DNA sequence data.

As described in Chapter II, PCR provided the most sensitive detection for known and unknown phytoplasmas. This technique was successfully used to diagnose a new phytoplasmal disease, monarda yellows (Chapter V). The nucleic acids were extracted from infected monarda and used as template for PCR amplification. A 16S rDNA fragment was amplified from diseased samples but not from healthy one. RFLP analysis revealed that monarda yellows phytoplasma belongs to the AY subclade and 16Sr I-A subgroup. This was the first report that monarda yellows was caused by a phytoplasma.

In Chapter V, nested-PCR assays were used not only for increasing the sensitivity and specificity in detection of phytoplasmas in woody plant hosts in which phytoplasma titers are usually low and in which the phytoplasmas are often unevenly distributed, but also for increasing the yield of specific PCR products so as to provide a sufficient aomunt of 16S rDNA for further confirmation of phytoplasma identities by RFLP analyses of amplified 16S rDNA sequences. To detect all the possible constituent phytoplasmas in mixed-infections of single plants, it is recommended to perform a series of nested-PCR assays using universal primers and group- or disease-specific primers (Lee *et al.*, 1994). For instance, a less frequently detected phytoplasma isolate which was genetically different from a prevalent isolate could only be identified by nested PCR using universal, group- and pathogen-specific primers (Marcone *et al.*, 1996).

In Chapter VI, it was established that phytoplasma-free clones were obtained by a combination of meristem culture after heat-treatment with PCR-based screening. The infected paulownia tissue cultures were subjected to heat-treatment at 35 ^{o}C in growth chambers for 5 weeks. The meristems of shoots were excised from treated tissue cultures

and were cultured at 25 °C. The results showed that 94% of the treated tissue cultures were free of phytoplasmas as screened by PCR.

There are no practical control measures proven to be effective for phytoplasmal diseases. All chemical control measures failed to cure phytoplasmal diseases. Thus, non-chemical control measures such as planting clean shocks, controlling insect vectors, eliminating alternate plant hosts etc. are becoming increasingly important in controlling phytoplasmal diseases. However, many of these control measures have not been successfully put into practice. The reason for the failure is that highly specific, sensitive, and accurate detection methods have not been available for detecting the phytoplasmas in test samples. In many cases, even after the insect vectors and alternate plant hosts were completely controlled, target crop plants were still infected by phytoplasmas and the yield were not increased by any of the treatments (Thompson *et al.*, 1973) because the stocks contained phytoplasmas which would develop in the plants later. Therefore, sensitive and reliable methods for detection of phytoplasmas are essential for application of non-chemical measures to control phytoplasmal diseases. The highest sensitive detection methods provided in Chapter II and V make it possible to obtain truly phytoplasma-free plants (Chapter VI).

VII. 2. CONCLUSIONS

A series of studies on phytoplasmal diseases were carried out in this research. PCR and nested-PCR provided the most sensitive method for phytoplasma detection. A minimum of 16 pg of total nucleic acids extracted from infected paulownia plants was sufficient for this detection. For the first time, it was reported that the concentration of

phytoplasmas in tissue cultures was five times higher than that detected in the host plant under greenhouse conditions. Micropropagation of infected host plants and microwave treatment of infected tissues were very useful for maintenance of phytoplasmas *in vivo*. The evidence that the brief microwave treatment of infected tissues did not affect 16S rDNA sequences was reported for the first time. PaWB phytoplasma isolates in Asia were very similar but different from JWB phytoplasma isolates which were also very closely related to each other on the basis of analyses of 16S rDNA sequences. The results revealed that phytoplasma diseases in diverse geographic locations were caused by an identical phytoplasma strain. Jujube witches'-broom phytoplasma strain was identified as a member of 16S rRNA group V (EY and related phytoplasmas) but different from EY phytoplasma. A simple and reliable method was developed for DNA sequencing. DNA sequence data have revealed the highly conserved nature of phytoplasmal 16S rRNA genes. Molecular analyses of less conserved gene sequences such as a ribosomal protein gene operon have been recommended for revealing a finer level of differentiation among strains in each phytoplasma groups. Using PCR techniques, monarda yellows, for the first time, was found to be associated with a phytoplasma which was identified as a member of 16Sr I-A subgroup. Most importantly, the establishment of phytoplasma-free clones by a combination of heat treatment, meristem culture, and PCR screening achieved in this study provides a practical method for controlling phytoplasma diseases in the future..

VII. 3 FUTURE PERSPECTIVES

The following projects are suggested for future research to generate detailed information for controlling phytoplasmal diseases:

1. Survey of alternate plant hosts and insect vectors. Phytoplasmas are primarily transmitted by insects. The alternate plant hosts of phytoplasmas act as sources of inoculum in spring next year. The results of this study will provide useful information for non-chemical control of phytoplasma diseases

2. Cross transmission of related phytoplasmal diseases. RFLP analyses and 16S rDNA sequence data have revealed that some phytoplasmas occurring in different plant species or different locations were very closely related or identical to each other, sharing the same 16S rDNA sequences. Cross transmission of related phytoplasmas combined with further analyses with modern molecular techniques will yield evidence for clarification of their relationships.

3. Epidemiology of phytoplasmal diseases. Until now, only a few studies have been conducted in this area. The unknown epidemiology of phytoplasma diseases has greatly limited the understanding of effective disease control measures. If the host range, insect vectors, and insect migration are clearly understood, the prediction of new disease out-break and spread can be made to formulate effective disease control measures.

4. Mechanisms of phytoplasma infection. Recent studies suggest that phytoplasma infection can cause a disorder of phytohormone in infected plants and increase the activities of certain enzymes such as peroxidase, polyphenol oxidase. However, many molecular mechanisms of phytoplasma infection still remain unclear. The

understanding of phytoplasma infection at the level of molecular biology, genetics and biochemistry will make it possible to answer certain specific questions such as whether or not phytoplasma DNA incorporates the expression of host DNA and how it changes the host gene functions.

6. Replication mechanisms of phytoplasmas. The multiplication of phytoplasmas apparently occur in infected plants and insect vectors. The phenomena of binary division was observed in ultrathin sections by electron microscopy. If the mechanisms of phytoplasma replication is understood, it will be a significant contribution to our understanding of phytoplasma pathology at the molecular level and assist in successful culture of phytoplasma *in vitro* as well as in understanding of the infection mechanisms.

VII. 5 REFERENCES

Ahrens, U., and Seemüller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82, 828-832.

Alma, A., Davis, R.E., Vibio, M., Danielli, A., Bosco, D., Arzone, A., and Bertaccini, A. 1996. Mixed infection of grapevines in northern Italy by phytoplasmas including 16S rRNA RFLP subgroup 16SrI-B strains previously unreported in this host. Plant Dis. 80, 418-421.

Chen, K.H., Guo, J.R., Wu, X.Y., Loi, N., Carraro, L., Guo, Y.H., Chen, Y.D., Osler, R., Pearson, R., and Chen, T.A. 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows disease agent. Phytopathology 83, 915-922.

Deng, S., Forster, R.J., Hiruki, C., and Teather, R.M. 1993. Simultaneous amplification and sequencing of genomic DNA (SAS): sequencing of 16S rRNA genes using total genomic DNA from *Butyrivibrio fibrisolvents*, and detection and genotyping of nonculturable mycoplasmalike organisms directly from total DNA isolated from infected plants. J. Microbiol. Methods 17, 103-113.

Deng, S., and Hiruki, C. 1990. Enhanced detection of a plant pathogenic mycoplasmalike organisms by polymerase chain reaction. Proc. Jpn. Acad. 66B, 140-144.

Gundersen, D.E., Lee, I.-M., Rehner, S.A., Davis, R.E., and Kingsbury, D.T. 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas); a basis for their classification. J. Bacteriol. 176, 5244-5254.

Gundersen, D.E., Lee, I.-M., Schaff, D.A., Harrison, N.A., Chang, C.J., Davis, R.E., and Kingsbury, D.T. 1996. Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int. J. Syst. Bacteriol. 46, 64-75.

Jarausch, W., Lansac, M., and Dosba, F. 1996. Long-term maintenance of nonculturable apple-proliferation phytoplasmas in their micropropagated natural host plant. Plant Pathol. 45, 778-786.

Kirkpatrick, B., Smart, C., Gardner, S., Gao, J.-L., Ahrens, U., Mäurer, R., Schneider, B., Lorenz, K.-H., Seemüller, E., Harrison, N., Namba, S., and Daire, X. 1994. Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA space sequences. IOM Letters 3, 228-229.

Lee, I.-M., Davis, R.E., Sinclair, W.A., DeWitt, N.D., and Conti, M. 1993a. Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in USA and Italy by means of DNA probes and polymerase chain reaction. Phytopathology 83, 829-833.

Lee, I.-M., Gundersen, D.E., Hammond, R.W., and Davis R.E. 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology 84, 559-566.

Lee, I.-M., Hammond, R.W., Davis, R.E., and Gundersen, D.E. 1993b. Universal amplification and analysis of pathogen 16S rDNA for classification and identification mycoplasmalike organisms. Phytopathology 83, 834-842.

Marcone, C., Ragozzino, A., Schneider, B., Lauer, U., Smart, C.D., and Seemüller, E. 1996. Genetic characterization and classification of two phytoplasmas associated with spartium witches'-broom disease. Plant Dis. 80, 365-371.

Mäurer, R., Seemüller, E., and Sinclair, W.A. 1993. Genetic relatedness of mycoplasmalike organisms affecting elm, alder, and ash in Europe and North America. Phytopathology 83, 971-976.

Namba, S., Oyaizu, H., Kato, S., Iwanami, S., and Tsuchizaki, T. 1993. Phylogenetic diversity of phytopathogenic mycoplasmalike organisms. Int. J. Syst. Bacteriol. 43, 461-467.

Prince, J.P., Davis, R.E., Wolf, T.K., Lee, I.-M., Mogen, B.D., Dally, E.D., Bertaccini, A., Credi, R., and Barba, M. 1993. Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. Phytopathology 83, 1130-1137.

Schaff, D.A., Lee, I.-M., and Davis, R.E. 1992. Sensitive detection and identification of mysoplasmalike organisms by polymerase chain reaction. Biochem. Biophys. Res. Comm. 186, 1503-1509.

Sinclair W.A., Griffiths, H.M., and Davis, R.E. 1996. Ash yellows and lilac witches'-broom: phytoplasmal diseases of concern in forestry and horticulture. Plant Dis. 80, 468-475.

Thompson, L.S., Cutcliffe, J.A., Gourley, C.O., and Murray, R.A. 1973. Evaluation of several insecticides for control of strawberry green petal disease. Can. Plant Dis. Survey 53, 16-18.